EXHIBIT N

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Effects on 4080 Rats of Chronic Ingestion of *N*-Nitrosodiethylamine or *N*-Nitrosodimethylamine: A Detailed Dose-Response Study[1]

Richard Peto, Richard Gray, Paul Brantom, and Paul Grasso[2]

ICRF Cancer Studies Unit, Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Oxford OX2 6HE [R. P., R. G.], and BIBRA Toxicology International, Woodmansterne Road, Carshalton, Surrey [P. B., P. G.], United Kingdom

Abstract

Four thousand eighty inbred rats were maintained from weaning on various different concentrations of *N*-nitrosodiethylamine (NDEA) or *N*-nitrosodimethylamine (NDMA). The principal aim was to characterize the dose-response relationship for the effects of these agents on esophageal cancer (NDEA) or on various types of liver cancer (NDEA and NDMA), although NDEA also caused a few tumors of the nasopharynx and NDMA also caused a few tumors of the lung.

The numbers of tumors of mesenchymal and Kupffer cells in the liver were too few to allow easy characterization of the dose-response relationships, and although NDMA induced large numbers of bile duct neoplasms, NDEA did not. Thus, the four principal dose-response relationships studied were of NDEA on esophageal or liver cells and of NDMA on bile duct or liver cells.

At doses sufficiently high for the median time to death from the disease of interest to be estimated, relationships were observed of the general form

$$(\text{Dose rate}) \times (\text{median})^n = \text{constant}$$

where n was about 2.3 for the first three relationships and about 1 for the last one (NDMA on liver cell tumors).

By contrast, at doses sufficiently low for longevity to be nearly normal (median survival about 2.5 years), there remained no material dependence on the dose rate of the age distribution of the induced neoplasms. At these low dose rates, the number of liver (but not of esophageal) neoplasms induced by treatment was simply proportional to the dose rate. This finding is not surprising, since the background incidence of liver (but not of esophageal) neoplasms was appreciable. The linear relationship observed at low dose rates (below 1 ppm) suggests that under these experimental conditions, among rats allowed to live their natural life span, a dose of 1 ppm of NDEA or NDMA in the drinking water will cause about 25% to develop a liver neoplasm, a dose of 0.1 ppm will cause about 2.5% to do so, and a dose of 0.01 ppm will cause about 0.25% to do so, etc., with no indication of any "threshold." (At these low dose rates, the incidence of liver neoplasms appears likely to exceed greatly that of esophageal neoplasms.)

In addition, even quite low dose rates of the test agents caused a variety of nonneoplastic liver abnormalities (*e.g.*, hyperplastic nodules, or shrinkage of hepatocytes) at a frequency roughly proportional to the dose rate.

Introduction

In an experiment on 4080 inbred rats, various concentrations of NDMA[3] or of NDEA were present in the drinking water, starting at about 6 weeks of age and continuing throughout life. Given in this way, both NDEA and NDMA are known to have a strong effect on liver tumor onset rates (1), and NDEA (but not NDMA) to have a strong effect on esophageal tumor onset rates (1). The principal aim of this experiment was to characterize the three dose-response relationships for these three strong effects.

However, it was not known which (if any) other anatomic sites would be measurably affected by these agents given in this way. In view of its large size and long duration it is possible that in the present study carcinogenic effects at sites other than the liver or esophagus that would be too weak to be detected by smaller experiments might be clearly evident. The present report therefore includes not only a basic description of the three main dose-response relationships [which will be characterized in more detail in a separate report (2)] but also a battery of tests of the statistical significance of the trends with respect to dose of the numbers of tumors observed at each of several different anatomic sites.

Because many anatomic sites may be considered and because for each there are up to 4 dose-response relationships that can be examined (which will be referred to subsequently as A, B, C, and D, where A is a NDEA male, B is a NDEA female, C is a NDMA male, and D is a NDMA female), each based on over 1000 rats, some false positive trends can be expected by chance alone. Consequently, unless a test for trend is very highly statistically significant it can be accepted as good evidence for a real effect only if supported by some ancillary evidence, such as a highly significant dose-response relationship for some type of hyperplastic, biochemical, or other effect of the relevant test agent at the anatomic site of interest.

Methods

1. Methods of Allocation, Husbandry, and Postmortem Examination

Ten successive "batches" of inbred weanling Colworth rats, each including more than 204 males and 204 females, were delivered at approximately 2-week intervals. (These are Wistar rats, derived originally from the Unilever breeding colony.) Two hundred four males and 204 females that were judged to be normal in weight and appearance were allocated between treatments, using computerized randomization lists, in numbers indicated by division by 10 of the numbers of animals specified in Table 1. (Only *after* each animal had been picked out as appearing normal was the person selecting it told of the treatment allocation.) Following a subsequent acclimatization period of about 2 weeks, *i.e.*, at approximately 6 weeks of age, treatment began, involving various concentrations of NDEA or NDMA in the drinking water (Table 1). After 12 months of treatment the survivors of Batch 1, and after a total of 18 months of treatment the survivors of Batch 2, were all sacrificed. Apart from this, no animals were sacrificed unless they were apparently dying or were thought to have palpable liver lumps.

Details of animal husbandry and of the preparation and disposal of nitrosamine solutions are given in "Appendix 3." Noteworthy features include: (*a*) All animals were palpated

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[1] This experiment was commissioned by the Ministry of Agriculture, Fisheries and Food (MAFF) in consultation with the Department of Health and was executed at BIBRA and analyzed at Oxford.

[2] Retired.

[3] The abbreviations used are: NDMA, *N*-nitrosodimethylamine; NDEA, *N*-nitrosodiethylamine.

Table 1 *Initial distribution of 4080 weaning rats among treatment groups*

Treatment group	Nitrosamine dose[a] (concentration, in ppm by volume in the drinking water, to be maintained from approximately 6 wk of age onwards[b])	Male			Female		
		NDEA	Control	NDMA	NDEA	Control	NDMA
1	0		240			240	
2	0.033	60		60	60		60
3	0.066	60		60	60		60
4	0.132	60		60	60		60
5	0.264	60		60	60		60
6	0.528	60		60	60		60
7	1.056	60		60	60		60
8	1.584	60		60	60		60
9	2.112	60		60	66[c]		60
10	2.640	60		60	60		60
11	3.168	60		60	60		60
12	4.224	60		60	60		60
13	5.280	60		60	60		60
14	6.336	60		60	60		60
15	8.448	60		60	60		60
16	16.896	60		60	54[c]		60
All doses		900	240	900	900	240	900
		2040 males			2040 females		

[a] Dose in mg/kg/day may be obtained by dividing ppm by a factor of 20 or so; see "Materials and Methods," Section 8.

[b] For a maximum of 12 months for 10% of the animals, then sacrifice. For a maximum of 18 months for 10% of the animals, then sacrifice. For a maximum of ~3.5 years for 80% of the animals, *i.e.*, lifelong.

[c] Due to an administrative error at the time of randomization, six females that should have been allocated high-dose NDEA were actually allocated a more moderate dose, and once this error was discovered it was decided to maintain them on this more moderate dose indefinitely.

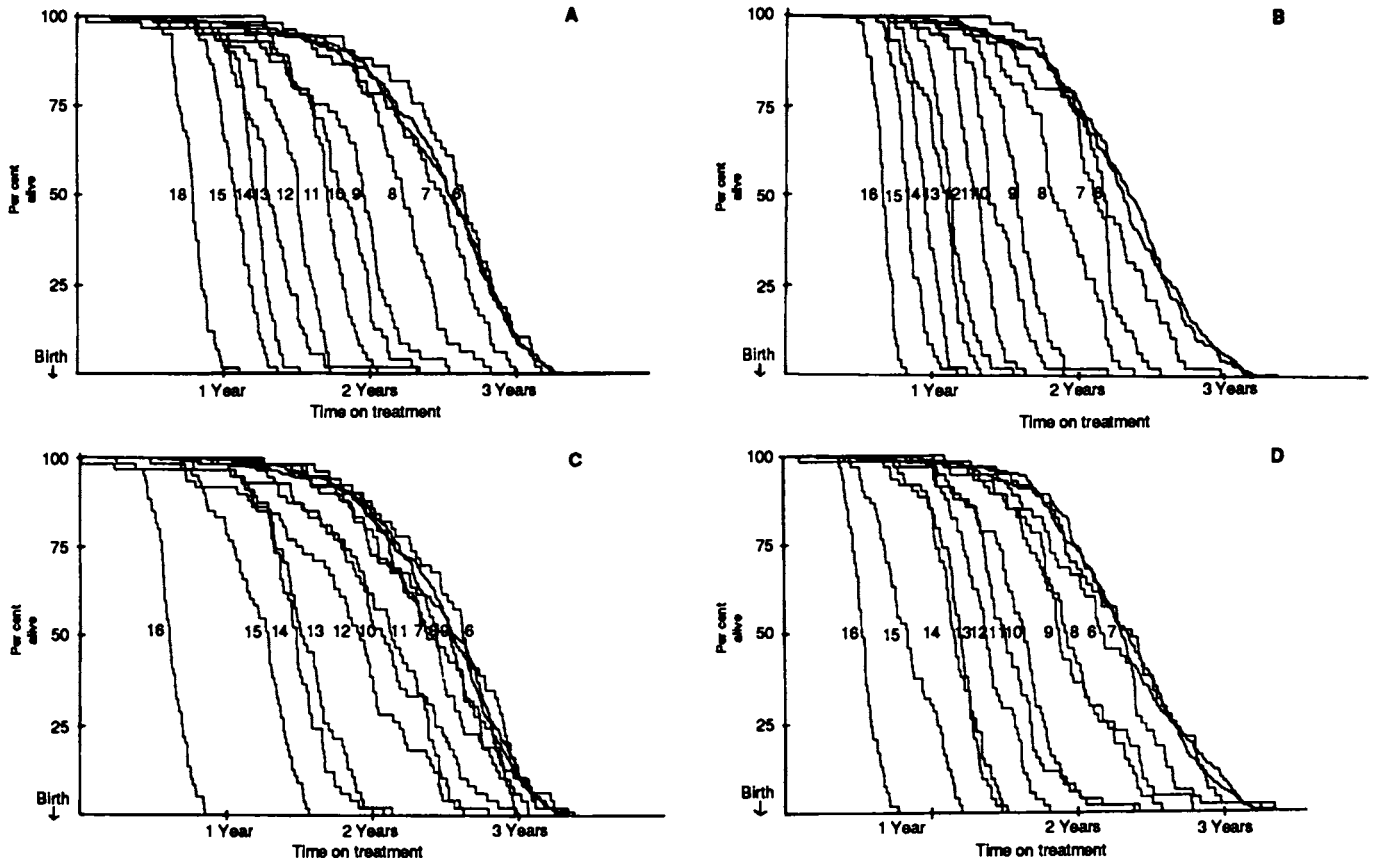


Fig. 1. *A*, NDEA-treated and control males; Survival. The number by each line indicates the corresponding treatment level (based only on rats that died without diaphragmatic hernia or total autolysis or cannibalism; adjusted actuarially for the scheduled sacrifice of one-tenth of the rats after 12 and after 18 months of treatment), 16 being the highest and 1 being control. For clarity, level 2–5 are merged. *B*, NDEA-treated and control females; *C*, NDMA-treated and control males; *D*, NDMA-treated and control females.

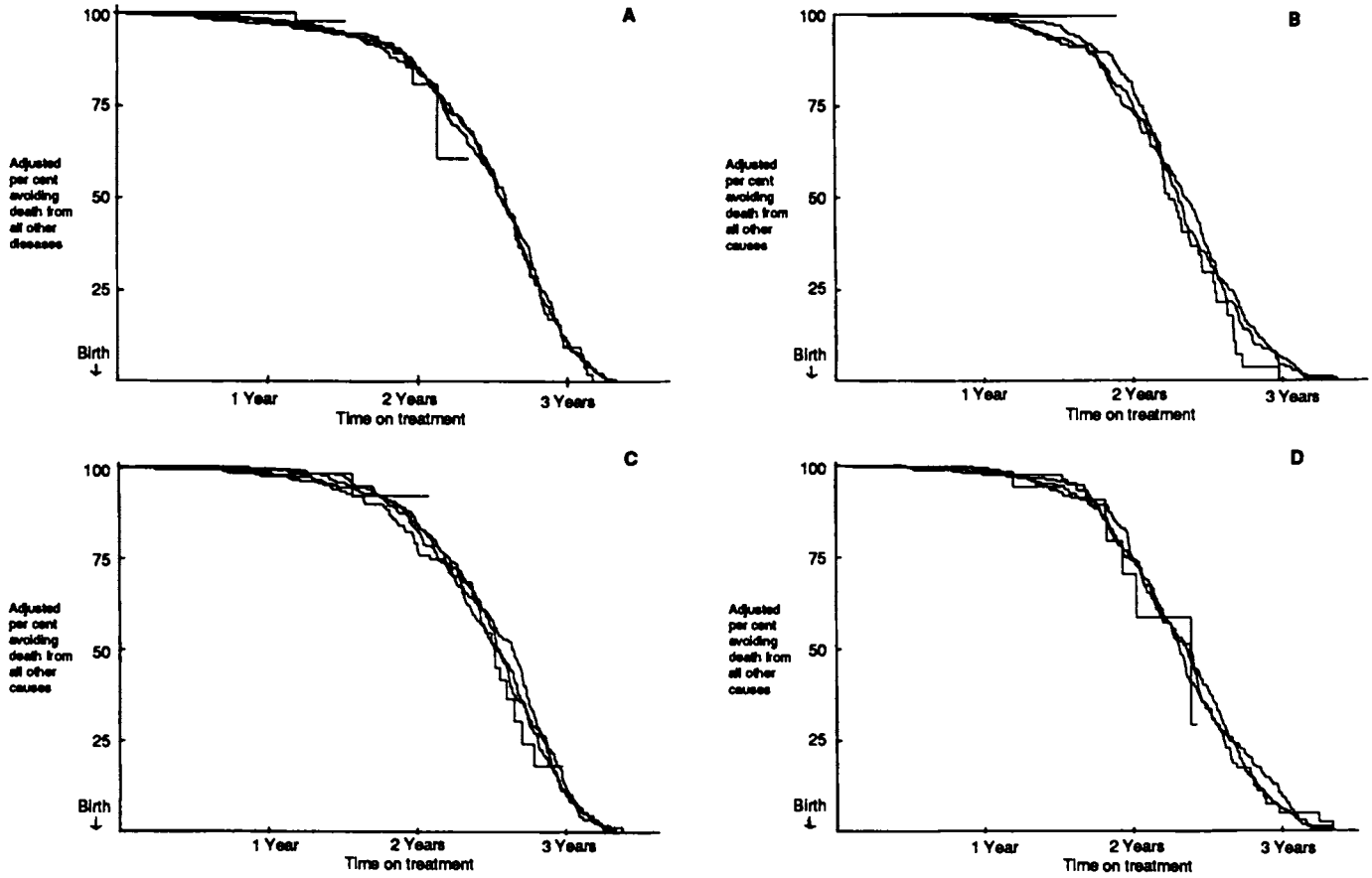


Fig. 2. Mortality from diseases other than of liver or esophagus. Kaplan-Meier estimates of the percentages that would have remained alive at various times if deaths from scheduled sacrifice, oesophageal disease or liver disease (including sacrifice following palpation of an apparent liver abnormality) were prevented. Groups 2–5, 6–9, 10–13, and 14–16 have been pooled. *A*, NDEA dose, males; *B*, NDEA dose, females; *C*, NDMA dose, males; *D*, NDMA dose, females.

weekly, and those with "clearly palpable" liver abnormalities were immediately sacrificed and autopsied. (*b*) The chief aim of autopsy was to record and section for histology any macroscopically evident possibly neoplastic lesions, and to help determine which, if any, such neoplasms were responsible for death. Only a few sections of apparently normal liver and esophagus were scheduled to be taken routinely. (*c*) Survival was remarkably good (up to about 3.5 years in the low-dosed groups), allowing demonstration of treatment effects that would have been too weak to have been seen in a standard 2-year study. Total mortality is described in Fig. 1, in which, for clarity, treatment groups 2–5 (dose levels 33, 66, 132, and 264 ppb) have been merged, since their four patterns of survival were so similar (and so similar to that of the control group) that separate display of them would have been visually confusing. In these lower-dosed groups and in the control group the animals survived well, to a median of 30 months of age for the females (28 months on treatment) and 32 months of age for the males (30 months on treatment). This longevity suggests that the standards of animal husbandry were generally adequate.

2. *Statistical Methods: General Description*

Unbiased correction for the effects of intercurrent mortality on expected tumor yields is advisable in many long-term carcinogenesis experiments and is peculiarly important in the present one because (*a*) the dependence of longevity on treatment is unusually strong and (*b*) the numbers are unusually large, so that for tumors other than of the liver or esophagus even small residual biases in the statistical method might change what should be a nonsignificant result into a highly significant one, or *vice versa*. The statistical methods required for such data have only recently become standardized (3); thus in "Methods," Section 3, the principles that underlie them are reviewed at some length for readers who may be new to them. Many readers, however, should (at least initially) skip directly over this lengthy material to "Methods," Section 4, which summarizes it.

The progressively worse survival in the higher-dosed groups is, of course, due principally to death from nitrosamine-induced tumors of the esophagus or liver (including a few tumors of the Kupffer cells), to the sacrifice of animals that were severely ill from these diseases, or to the sacrifice of animals in which liver abnormalities were thought to have been palpated. If we define these to be the "strongly dose-related" causes of death, then the age-specific death rates from the aggregate of all remaining causes[4] except scheduled sacrifice were not materially related to treatment (Fig. 2). This suggests that although some of the remaining tumor types may perhaps be significantly affected by treatment, any such effects will be far less gross than for tumors of the liver or esophagus. Consequently, reliable demonstration of them may require particularly careful statistical methodology; otherwise biases or random errors might conceal (or masquerade as) a moderate effect of treatment on some particular type(s) of tumor. There are three main aspects of these data that can be simplified [*sic*] by appropriate statistical treatment.

The first involves graphic presentation of the overwhelmingly significant dose-response relationships (*e.g.*, esophageal neo-

[4] It would have made no difference to Fig. 2 if tumors of the nasopharynx had been included among the diseases that were "strongly dose-related," because no such tumors killed their hosts.

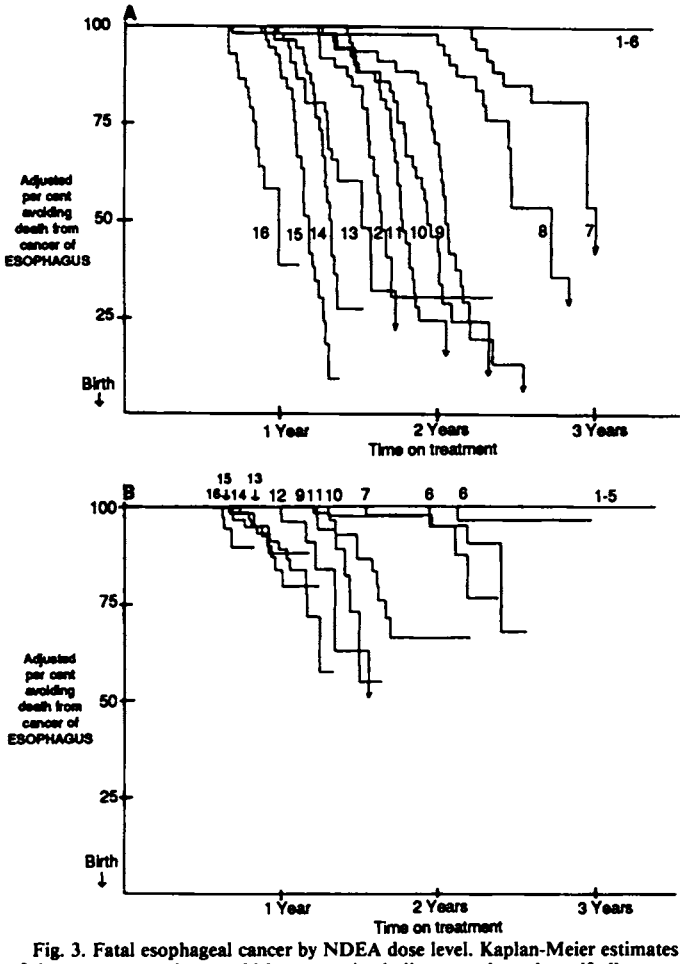


Fig. 3. Fatal esophageal cancer by NDEA dose level. Kaplan-Meier estimates of the percentages that would have remained alive at various times if all causes of death were prevented except for the condition of interest. The number by each line indicates the corresponding treatment level, 16 being the highest and 1 being control. *Downward arrows*, times at which only one animal remained in a particular group, and it died of the condition of interest. *A*, males; *B*, females.

plasms among NDEA-treated males: see Fig. 3*A*) by "Kaplan-Meier" graphs, such as in Fig. 2. These illustrate the proportions of animals that would still be alive at various times if, hypothetically, all causes of death other than the disease of interest were eliminated (or, more formally, if the total death rate at each age were equal to the death rate at that age that was actually observed from the disease of interest). Kaplan-Meier graphs essentially describe *cumulative mortality* and take no account of incidental (nonfatal) neoplasms. For some particular disease the Kaplan-Meier *median* in a particular treatment group (*i.e.*, the time at which the Kaplan-Meier graphs for that group first cross the 50% mark) can be a useful index of the cumulative mortality from that particular cause, corrected for the effects of mortality from all other causes, and the relationship of these medians to the dose rate can usefully characterize a *strong* dose-response relationship for *fatal* tumors.

The second use of statistical treatment to simplify interpretation of these data involves allowance for the effects of the premature extinction of certain treatment groups on their expected tumor yields. The third, which is useful chiefly for types of tumor for which the dose-response relationship is not overwhelmingly strong, involves the use of tests for *trend* (rather than multiple pairwise comparisons of particular treatment groups with each other or with the control group).

Too statistical an approach to such data is not desirable, especially since many, and perhaps even most, of the scientists and administrators to whom the results are relevant have no particular affinity for statistics. However, the proper use of *observed and expected* numbers, together with tests for *trend*, can be helpful when such a mass of data is to be interpreted, and readers who are willing to try to use these tools will not find them difficult, no matter how little statistical background they may have.

3. *Statistical Methods: Detailed Rationale*[5]

3A. Comparison of Observed with Longevity-corrected Expected Number. The unusually good survival into extreme old age allowed a greater number of spontaneous tumors to develop than would have been possible in a standard 2-year experiment and thus gave more opportunity for the manifestation of any weak carcinogenic effects that treatment may have on sites other than the liver or esophagus. In the higher-dosed groups, however, early mortality from tumors of the liver or esophagus was so severe (see Fig. 1) that there might not have been enough time for tumors to develop at other anatomic sites. Thus, even if for some particular such tumor type, administration of the higher dose rates of nitrosamines doubled its onset rate among animals of a given age, the crude percentages of animals that developed it might actually be *lower* in the higher-dosed animals, simply because the increased onset rate was outweighed by the shorter life span. Consequently, simple examination of the crude percentages of tumor-bearing animals is not a reliable way of discovering which (if any) of these other types of tumor were caused by treatment. This difficulty can be circumvented if (for each particular category of neoplasm) O, the number of animals in each treatment group that were observed to have that neoplasm, is compared with E, the number that would have been expected to have done so if the onset rates of such neoplasms *among animals of a given age* were unrelated to treatment (3).

Table 2 gives, in some detail, one particular example of such a comparison, to illustrate the general method. Table 2 obviously *appears* somewhat more complicated than a simple table of percentages, but the essence of it is simply the comparison of the observed and expected numbers in each group, which is not really any more complex than examining a table of percentages.

Indeed, because comparison of observed with expected numbers obviates the need to worry about the effects of differences in survival on tumor yields, it could be argued that it is actually a considerably less complex procedure than comparisons of "crude" percentages. For example, it is relatively straightforward to interpret the observation that in the top 8 groups in Table 2 a total of 10 uterine tumor-bearing animals were observed where 12.0 would (in the statistical sense of the word) have been expected; these two numbers obviously do not differ significantly from each other, and thus the data offer no evidence that treatment with these doses of NDEA has either enhanced or inhibited the processes of uterine carcinogenesis (except in the trivial sense of preventing uterine cancer by killing the animals before they reached the ages where it typically occurs). However, without calculation of the expected numbers (or some other appropriate statistical procedure), how could one possibly come to a reliable biological interpretation of the

[5] NOTE: Many readers may, at least initially, pass over Section 3 to Section 4, which summarizes it.

Table 2 *Observed and expected numbers, with no significant trend: neoplasms (benign or malignant, fatal or incidental) of the uterus among female controls and female rats treated with NDEA*

Treatment group	NDEA concentration[a] (ppm)	Initial group size	*O* Observed no. with such neoplasms	*E* Expected no. with such neoplasms[b]	Ratio (*O*/*E*)	Difference (*O* − *E*)	Product [dose × (*O* − *E*)]
1	0	240	68 (28)[c]	66.9	1.0	+1.1	0.0
2	0.033	60	14 (23)	20.3	0.7	−6.3	−0.2
3	0.066	60	19 (32)	16.7	1.1	+2.3	+0.2
4	0.132	60	28 (38)	15.0	1.5	+8.0	+1.1
5	0.264	60	17 (28)	18.4	0.9	−1.4	−0.4
6	0.528	60	12 (20)	13.5	0.9	−1.5	−0.8
7	1.056	60	12 (20)	17.0	0.7	−5.0	−5.3
8	1.584	60	13 (22)	8.2	1.6	+4.8	+7.6
9	2.112	66[a]	5 (8)	7.6	0.7	−2.6	−5.5
10	2.640	60	2 (3)	2.1	0.9	−0.1	−0.4
11	3.168	60	2 (3)	1.6	1.2	+0.4	+1.2
12	4.224	60	Total 1 (2)	Total 0.5	Total 2.2	Total +0.5	+2.3
13	5.280	60	Total size = 480[d] 0 (0)	*O* = 10 (2%)[d] 0.1	*E* = 12.0[d] 0.0	*O*/total *E* = 0.8[d] −0.1	−0.5
14	6.336	60	0 (0)	0.1	0.0	−0.1	−0.6
15	8.448	60	0 (0)	0.02	0.0	−0.02	−0.2
16	16.896	54[a]	0 (0)	0	0.0	0.0	0.0
Total (all doses)		1140 females	188 (16)	188.0	1.0	0.0	*T* = −1.5 SE (*T*) = ±9.0[e] *z* = *T*/SE (*T*) = −0.17[f]

[a] See Table 1, Footnote *a*.

[b] Each of these 16 expected numbers was calculated, as described in the IARC report (3), by addition of 4 expected numbers derived from (i–ii) analysis of the findings at the 12- and 18-month scheduled sacrifice, (iii) a death rate analysis of all fatal or probably fatal uterine tumors, and (iv) a prevalence analysis of incidental uterine tumors among animals that did not undergo scheduled sacrifice, that did not (or probably did not) die of uterine tumors, and whose abdomens arrived at autopsy largely intact (*i.e.*, free of gross autolysis or cannibalism).

[c] Numbers in parentheses, percentage.

[d] These totals relate to only the top 8 groups, where early deaths, chiefly due to treatment-induced tumors of the liver or esophagus, caused most animals to die before the ages at which spontaneous uterine malignancies tend to occur. This reduces both the observed and the expected numbers of such malignancies in the top dose groups to such small values that the ratios *O*/*E* in individual groups become much less reliable than in the lower-dosed groups, where many animals lived on into old age.

[e] SE(*T*) denotes the standard error that *T*, the total of the 16 products, would have if treatment did not affect the age-specific onset rate of the disease of interest. SE(*T*) is calculated as described in IARC (3); for details, see "Appendix 1." For this tabulated relationship between NDEA dose and uterine malignancies, *T* is actually slightly negative; thus these data offer no evidence that the onset rate of uterine malignancies among female rats of a given age is positively affected by NDEA, because, if it were, *T* would tend to be positive, not negative. Note that if treatment were without any effect on the age-specific onset rates of the disease of interest then *T* would differ only randomly from zero, having an expectation of exactly zero; consequently, the probability that *T* would by chance alone exceed 1.96 SE(*T*) must be about 0.025. Positive values of *T* that are more than double SE(*T*) therefore do suggest a real carcinogenic effect, while values of *T* that are more than triple SE(*T*) virtually exclude the possibility that chance alone is responsible for the observed association between dose and disease. We shall use the symbol *z* to denote the ratio of *T* to SE(*T*) and shall regard values of *z* above 3 as being extremely strong evidence for a real effect of treatment (but see "Appendix 1").

[f] Not significant.

Table 3 *Observed and expected numbers, with highly significant trend: neoplasms (benign or malignant, fatal or incidental[a]) of the esophagus among male controls and male rats treated with NDEA*

Treatment group	NDEA concentration (ppm)	Initial group size	No. of animals with such neoplasms: *O* Observed	*E* Expected	Ratio (*O*/*E*)	Product [dose × (*O* − *E*)]
1	0	240	0 (0)[b]	110.8	0.0	0.0
2	0.033	60	0 (0)	28.4	0.0	−0.9
3	0.066	60	0 (0)	29.2	0.0	−1.9
4	0.132	60	0 (0)	28.9	0.0	−3.8
5	0.264	60	0 (0)	26.1	0.0	−6.9
6	0.528	60	3 (5)	28.7	0.1	−13.6
7	1.056	60	16 (27)	25.7	0.6	−10.2
8	1.584	60	32 (53)	23.8	1.3	+13.0
9	2.112	60	37 (62)[c]	16.2	2.3	+43.9
10	2.640	60	45 (75)[c]	16.7	2.7	+74.7
11	3.168	60	48 (80)[c]	15.4	3.1	+103.3
12	4.224	60	41 (68)[c]	15.8	2.6	+106.4
13	5.280	60	49 (82)[c]	12.7	3.9	+191.7
14	6.336	60	46 (77)[c]	11.5	4.0	+218.6
15	8.448	60	47 (78)[c]	9.3	5.1	+318.5
16	16.896	60	46 (77)[c]	10.9	4.2	+593.0
Total (all doses)		1140	410	410.0	(1.0)	*T* = 1626 SE(*T*) = 52.9 *z* = *T*/SE(*T*) = 30.7

[a] Fatal and incidental neoplasms were analyzed separately, as in Table 4; then the results of the two analyses in Table 4 were added to obtain the results in Table 3.

[b] Numbers in parentheses, percentage.

[c] Percentages of tumor-bearing animals are not straightforwardly informative among high-dosed animals; see text.

observation that over 20% of the females in groups 1–8 developed uterine neoplasms, as against only 2% of those in groups 9–16?

3B. Use of Tests for Trend, Rather Than Separate Tests of Each Observed *versus* Its Corresponding Expected. Even if there were an exactly steady progression of risk with respect to dose,

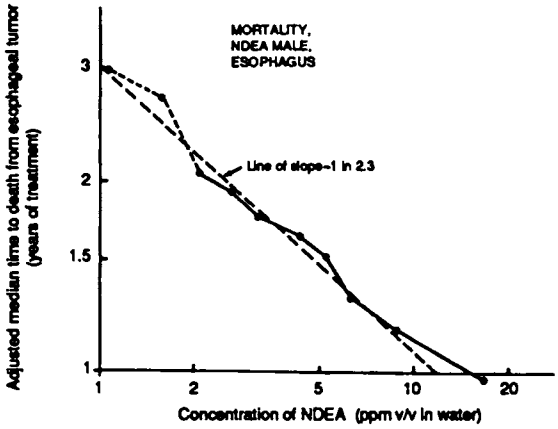


Fig. 4. Use of median time to death from cause of interest alone to describe dose response (for fatal esophageal neoplasms in NDEA-treated males; see Fig. 3*A*) *Broken lines* and *points* in *parentheses*, medians that were not reliable, being based on only a few neoplasms among only a few old animals; medians for still lower doses cannot be estimated directly. Multiplication of ppm v/v by 9.23 yields μmol NDEA/liter.

Table 4 *Separate analysis of "fatal" and of "incidental" lesions: observed and expected numbers of animals with esophageal neoplasm(s), malignant or benign, among control and NDEA-treated male rats*

N.B.: Table 3 was obtained by direct addition of the fatal and the incidental analyses in Table 4.

Treatment group	NDEA concentration (ppm)	Initial group size	Fatal[a] lesions				Incidental[b] lesions			
			Observed	Expected	Ratio (O/E)	Product [dose × ($O - E$)]	Observed	Expected	Ratio (O/E)	Product [dose × ($O - E$)]
1	0	240	0	58.9	0.0	0.0	0	51.9	0.0	0.0
2	0.033	60	0	15.0	0.0	−0.5	0	13.4	0.0	−0.4
3	0.066	60	0	15.8	0.0	−1.0	0	13.4	0.0	−0.9
4	0.132	60	0	15.4	0.0	−2.0	0	13.5	0.0	−1.8
5	0.264	60	0	13.6	0.0	−3.6	0	12.5	0.0	−3.3
6	0.528	60	0	15.5	0.0	−8.2	3	13.2	0.2	−5.4
7	1.056	60	8	14.5	0.6	−6.9	8	11.2	0.7	−3.4
8	1.584	60	13	14.0	0.9	−1.6	19	9.8	1.9	+14.6
9	2.112	60	26	10.5	2.5	+32.7	11	5.7	1.9[c]	+11.2
10	2.640	60	27	10.0	2.7[c]	+44.9	18	6.8	2.7[c]	+29.6
11	3.168	60	29	8.8	3.3[c]	+64.0	19	6.6	2.9[c]	+39.3
12	4.224	60	18	6.4	2.8[c]	+49.0	23	9.4	2.4[c]	+57.4
13	5.280	60	23	4.9	4.7[c]	+95.6	26	7.8	3.3[c]	+96.1
14	6.336	60	22	3.8	5.9[c]	+115.3	24	7.8	3.1[c]	+102.6
15	8.448	60	28	2.4	11.7[c]	+216.3	19	6.9	2.7[c]	+102.2
16	16.896	60	16	0.6	25.7[c]	+260.2	30	10.2	2.9[c]	+334.5
Total (all doses)		1140	210	210.0	(1.0)	T = 854	200	200.0	(1.0)	T = 772
						SE(T) = ±25.6				SE(T) = ±46.3
						$z = T/\text{SE}(T)$ = 33.4				$z = T/\text{SE}(T)$ = 16.7

[a] Observed and expected numbers were calculated by the "death rate" method for deaths from esophageal neoplasms [*i.e.*, with contexts of observation recorded as "fatal" or "probably fatal"; IARC (3)].

[b] Among animals where the whole esophagus was examined following death from a cause other than an esophageal neoplasm, observed and expected numbers of esophageal neoplasm-bearing animals were calculated by the "prevalence" method (3).

[c] O/E ratios are not straightforwardly informative for *incidental* tumors among *high*-dosed animals; see text.

the play of chance might mean that there was not an exactly steady progression of observed outcome with respect to dose. To continue the foregoing example, even if there were 15 expected and 30 observed in the top 8 treatment groups, it might be that in only a few (or even in none) of these 8 individual groups did the observed number significantly exceed the expected number, and it would not be particularly surprising to find that in one or two of these groups the observed number was actually somewhat less than the expected number. This would not indicate that those particular dose levels were without carcinogenic effect but merely that the overall pattern is more scientifically informative than the outcome in any single group considered alone. For this reason, the statistical method of choice is to see whether there is any statistically significant overall *trend* with respect to dose in the differences $O - E$ between the observed and expected numbers.

In essence, a test for trend involves asking whether the large $O - E$ values tend to be more strongly associated with the large dose rates than would be anticipated by chance alone. If the answer is clearly "yes," then it is reasonable to infer that treatment has caused some cases of the disease of interest. Conversely, if the answer is clearly "no," then it is reasonable to infer that there is no material effect of treatment on the disease of interest, even if in one particular treatment group or other the number observed differs quite markedly from the number expected; *e.g.*, in groups 2, 4, 7, and 8 in Table 2 there are divergences between the observed and the expected numbers that, in view of the lack of any overall trend with respect to dose, are presumably largely or wholly artifacts of chance.

The arithmetic details of a test for trend are outlined in Table 2 [and are set out more fully in the IARC report (3)], but they need not be mastered in order to understand and to use the trend test effectively. Briefly, it suffices to know that three quantities are calculated.

The first quantity, which will be denoted by the letter T, is a quantity called the "trend test statistic," which would tend to be somewhat positive if treatment did cause the disease of interest but which otherwise would, by chance alone, be either slightly negative or slightly positive (with mean exactly zero and calculable standard error).

The second quantity, which will be denoted by the letter z, is the number of these standard errors by which T exceeds zero.[6] Examination of z can yield a useful rough guide as to whether T is larger than would be plausible by chance alone (*i.e.*, if treatment did not cause the disease of interest), because it can be shown that the probability that z will exceed a value of 2 by chance alone is only a few per cent, while the probability that z will exceed 3 by chance alone is generally only a few tenths of a per cent.

The third quantity, which will be denoted by the symbol $1P$, is the "one-tailed P value," *i.e.*, the probability (estimated, as described in "Appendix 1," by methods that are likely to be more reliable than a standard normal approximation) that z would by chance alone have exceeded the value it actually took. $1P$ is thus the probability of obtaining by chance alone a trend with respect to dose of the $O - E$ values that is at least as positive as that actually observed.

If $1P$ is extremely small (*e.g.*, when $z > 3$ or $z > 4$) then there should, of course, be little doubt that treatment has caused the disease of interest. Conversely, if $1P$ is large (*e.g.*, when $z < 1$ or $z < 2$) then clearly the data do not of themselves show that treatment can cause the disease of interest (which, although it is in principle impossible from such data to prove that treatment has precisely zero effect on the disease of interest, does mean that any such effect is probably not a large one). Finally, if $1P$ is of intermediate magnitude (*e.g.*, when $2 < z < 3$) then judgment as to how likely it is that treatment really did cause the disease of interest becomes more difficult and may depend

[6] That is, $z = T$ (null hypothesis standard error of T). Statistically trained readers may be interested to note that z is useful not only as a test statistic but also as a means toward the numerical description of the strength of the overall dose-response relationship. For, if the log hazard function is linearly related to the dose rate, then z^2/T is approximately equal to the first Newton-Raphson step from zero towards the maximum likelihood estimate of the slope of this linear relationship (3), at least for fatal tumors.

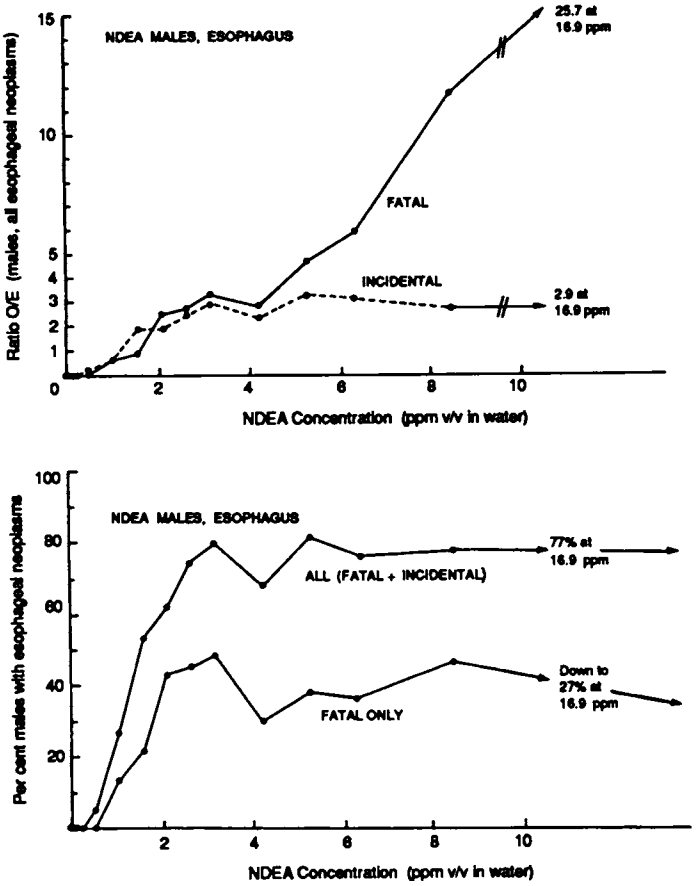


Fig. 5. Methodological difficulties in characterizing dose-response by *O/E* ratios or, particularly, by percentages affected. Methods exemplified by data for esophageal neoplasms in NDEA-treated males, from Table 4A. *Top*, fatal neoplasms, analyzed by "death rate" method: *consistent throughout dose range*; incidental neoplasms, analyzed by "prevalence" method: *inconsistent at high doses.* The 1-ppm data points are based on observation of 8 fatal and 8 incidental neoplasms, and at all higher doses higher numbers of each were observed. *Bottom*, all neoplasms, analyzed by percentages affected: *inconsistent at high doses*; fatal neoplasms, analyzed by percentages affected; *inconsistent at high doses.* Similar flatness above 2 ppm would have been observed if the analysis had been limited to the batches that were not scheduled for sacrifice at 12 or 18 months.

more on biological than on statistical considerations.[7]

Returning to the particular example of the uterine tumors in Table 2, the final column describes the method of calculation of the z value. The details of this calculation need not be followed, because it suffices to note that the calculated z value is near zero ($z = -0.17$). This confirms the impression that is conveyed by noticing in the observed and expected numbers (of animals with uterine neoplasms) in each group that there is no significant association between NDEA dose and the age-specific uterine tumor onset rate.

As a completely opposite example of the use of these methods, Table 3 gives details of an effect that is undoubtedly real. In Table 3 the z value is 30.7, a value so vastly in excess of 4 as to demonstrate beyond all reasonable doubt that NDEA causes esophageal tumors, a conclusion which in this instance would anyway have been overwhelmingly obvious from visual inspection, comparing the 16 observed with the corresponding 16 expected numbers in Table 3.

In both of these instances examination of the statistic z merely confirmed what would already have been obvious from casual inspection of the observed and expected numbers in Table 2 and 3. But in more marginal cases examination of z can be a useful aid to the judgment of how likely it is that an association between dose and disease that is only moderately clear reflects a cause-and-effect relationship and, corresponding, how likely it is that it reflects merely an artifact of chance.

The experimental results will therefore generally be summarized by citing, for various particular types of neoplasm, (*a*) the total number of animals found to have such neoplasms and (*b*) the statistics T and z, providing more detail (*e.g.*, a P value calculation, some Kaplan-Meier graphs, or a detailed ancillary tabulation of the observed and expected numbers of tumor-bearing animals in each group) only if the disease is of particular interest or if the value of z is large enough to be suggestive of a real causative effect.

3C. Numerical Description of a Dose-Response Relationship That Is Clearly Significant. For tumors of the liver and esophagus the chief need is not for tests of statistical significance, but rather for means of characterizing the dose-response relationship. In Table 3, the simple percentage of animals with esophageal malignancies does not exhibit the steady increase with increasing dose that the Kaplan-Meier graphs in Fig. 3*A* might lead one to expect. Indeed, beyond a concentration of 3 ppm of NDEA in the drinking water (at which 80% of males developed esophageal neoplasms) the percentage of males that developed such lesions remains approximately constant, and, as will be seen later, the percentage that died of such lesions actually decreases. This does not mean that higher doses inhibit the biological processes of esophageal tumorigenesis. (In fact, of course, higher doses accelerate the biological processes of both esophageal and liver tumorigenesis but, because the latter processes happen to be accelerated a little more than the former, as progressively higher dose rates of NDEA are tested, the percentage of animals that die of liver disease rises slightly at the expense of the percentage that die of esophageal disease.) It does mean, however, that if the relationship of NDEA dose to these biological processes is to be characterized at high dose levels, crude percentages of animals dying of or with the disease are of little value.

The best way to characterize such dose-response relationships is perhaps by a mathematical fit to the entire data of a suitable class of statistical distributions,[8] and a formal mathematical analysis of these data is reported in an accompanying paper (2).

A second approach is, as already noted, to relate the Kaplan-Meier "medians" for each treatment group at that group's dosage rate. Fig. 4 illustrates this approach for the Kaplan-Meier graphs of Fig. 3*A*, which described the cumulative mortality from esophageal neoplasms among NDEA-treated males. Although this method may sometimes be satisfactory, it describes only the data for fatal neoplasms and thus ignores all the information about neoplasms that are discovered "incidentally," at the autopsies of animals that have died of unrelated causes; and, depending on the type of neoplasm being studied, this may involve the loss of a substantial proportion of the information available from the experiment.

[7] For a fuller discussion of the proper interpretation of P values, see IARC (3). It would be completely wrong, especially in a large experiment such as this where many different types of tumor are being analyzed, to imagine that one "critical" significance level (*e.g.*, 0.05 or 0.01) can be adopted such that all trends that are "statistically significant" by this adopted criterion are necessarily real and such that all other trends can be dismissed. Unfortunately, even with the best statistical methods some "false negatives" and some "false positives" may still occur.

[8] For example, the "Weibull" distributions with dose-independent "shape" parameters, as recommended by Peto and Lee (4). (Even then, however, it may be more difficult to produce a satisfactory and reliable mathematical analysis of the dose-response relationships for the incidental than for the fatal neoplasms, and may be still more difficult to produce a satisfactory pooled analysis of the total tumor yield, fatal and incidental.)

Table 5A *Dose-response relationship for neoplastic or hyperplastic lesions of the esophagus in NDEA-treated males*

Treatment group	NDEA concentration (ppm)	Fatal lesions			Incidental lesions[a]		
		Expected	Observed M[b] + B	Ratio (*O/E*)	Expected	Observed M + B + H	Ratio (*O/E*)
1	0	58.9	0 + 0	0.0	64.7	0 + 0 + 0	0.0
2	0.033	15.0	0 + 0	0.0	16.7	0 + 0 + 0	0.0
3	0.066	15.8	0 + 0	0.0	16.7	0 + 0 + 0	0.0
4	0.132	15.4	0 + 0	0.0	16.8	0 + 0 + 0	0.0
5	0.264	13.6	0 + 0	0.0	15.6	0 + 0 + 1	0.1
6	0.528	15.5	0 + 0	0.0	16.4	0 + 3 + 0	0.2
7	1.056	14.5	3 + 5	0.6	14.1	0 + 8 + 6	1.0
8	1.584	14.0	9 + 4	0.9	12.5	5 + 14 + 3	1.8
9	2.112	10.5	21 + 5	2.5	7.9	2 + 9 + 4	1.9[c]
10	2.640	10.0	21 + 6	2.7	9.1	4 + 16 + 4	2.4[c]
11	3.168	8.8	24 + 5	3.3	8.6	6 + 14 + 3	2.6[c]
12	4.224	6.4	12 + 6	2.8	12.1	8 + 15 + 9	2.6[c]
13	5.280	4.9	16 + 7	4.7	10.2	9 + 17 + 8	3.3[c]
14	6.336	3.8	15 + 7	5.9	10.2	6 + 18 + 4	2.7[c]
15	8.448	2.4	20 + 8	11.7	9.1	5 + 14 + 6	2.8[c]
16	16.896	0.6	9 + 7	25.7	12.5	11 + 19 + 5	2.8[c]
Total (all doses)		210.0	150 + 60	(1.0)	253.0	56 + 144 + 53	(1.0)

[a] Among animals thought not to have died of disease of the target organ, and among whom intact target organ was examined postmortem.
[b] M, malignancy present somewhere in target organ; B, benign neoplastic disease but no malignancy anywhere in target organ; H, hyperplastic but no neoplastic disease (N.B.: Never fatal).
[c] *O/E* ratios do not characterize the dose-response relationship satisfactorily for incidental tumors in groups 9–16: see "Methods," Section 3C.

Table 5B *Dose-response relationship for neoplastic or hyperplastic lesions of the esophagus in NDEA-treated females*

Treatment group	NDEA concentration (ppm)	Fatal lesions			Incidental lesions[a]		
		Expected	Observed NK[b] + M + B	Ratio (*O/E*)	Expected	Observed M + B + H	Ratio (*O/E*)
1	0	16.7	0 + 0	0.0	72.4	0 + 0 + 0	0.0
2	0.033	4.1	0 + 0	0.0	18.0	0 + 0 + 0	0.0
3	0.066	4.3	0 + 0	0.0	18.8	0 + 0 + 0	0.0
4	0.132	4.2	0 + 0	0.0	18.4	0 + 0 + 0	0.0
5	0.264	4.4	0 + 0	0.0	17.6	0 + 0 + 0	0.0
6	0.528	4.1	0 + 1	0.2	18.4	0 + 2 + 0	0.1
7	1.056	4.1	1 + 3	1.0	17.3	1 + 14 + 6	1.2
8	1.584	3.6	3 + 0	0.8	17.6	5 + 13 + 5	1.3
9	2.112	3.4	1[b] + 10 + 1	3.5	15.2	6 + 14 + 3	1.5[d]
10	2.640	2.6	1 + 0	0.4	20.4	10 + 18 + 9	1.8[d]
11	3.168	2.1	4 + 2	2.9	18.5	11 + 25 + 7	2.3[d]
12	4.224	1.5	5 + 1	3.9	18.8	6 + 25 + 7	2.0[d]
13	5.280	1.3	9 + 2	8.5	17.4	9 + 24 + 3	2.1[d]
14	6.336	0.9	5 + 3	8.5	18.8	8 + 25 + 7	2.1[d]
15	8.448	0.6	2 + 1	4.8	21.3	10 + 23 + 15	2.3[d]
16	16.896	0.1	3 + 0	21.2	16.0	8 + 15 + 11	2.1[d]
Total (all doses)		58.0	1[b] + 43 + 14	(1.0)	345.0	74 + 198 + 73	(1.0)

[a] See Table 5A, Footnote *a*.
[b] NK, not known, due to loss of tissues.
[c] See Table 5A; Footnote *b*.
[d] Less reliable.

A third method that is somewhat cruder but far simpler, and which can be used either for fatal tumors, for incidental tumors, or for all tumors (fatal or incidental), is simply to calculate the ratio *O/E* for each group, and examination of the *O/E* values in Table 3 reveals a reasonably informative (to judge by its regularity) progression with increasing dose rate. However, for treatments that strongly affect longevity even a graph of *O/E* against dose is likely to be reasonably satisfactory only as a means of describing the relationship with dose of tumor *death* rates, or the prevalence rates of incidental tumors in the limited range of dose levels (1–8 or so, in this experiment) that are too low to affect longevity strongly. They are not particularly satisfactory for describing *incidental* tumor prevalences at the *higher* dose levels (~9–16).[9]

This limitation is, for example, quite obvious when the analyses of the fatal and the incidental male esophageal neoplasms in Table 4 are examined separately. These two sets of *O/E* ratios are plotted against the NDEA concentration in Fig. 5. Those for the *fatal* neoplasms suggest (correctly) that throughout the dose range above 2 ppm there is a steady escalation of carcinogenic effect with increasing dose rate. By contrast, the *O/E* ratios for the *incidental* neoplasms suggest (probably misleadingly) that there is not. For comparison, the even more misleading dependence on dose of the crude percentages of animals dying of or with esophageal neoplasms is also presented in Fig. 5.

Thus, in summary, for tumors of the liver or esophagus the *O/E* ratios provide useful descriptive statistics for characterizing yields of fatal neoplasms in all treatment groups (1–16), but

[9] This is, moreover, a difficulty of principle rather than a defect in one particular statistical method. As an extreme example, suppose that an increase in treatment level that brought forward the ages at autopsy following death from causes other than esophageal cancer also brought forward the age at onset of esophageal cancer, so that the prevalence of this disease among such autopsies was hardly affected by the increase in treatment level. It might then be impossible to distinguish, just from the data from such an experiment, between this real effect of treatment on the disease of interest and the opposite hypothesis that the prevalence of the condition of interest depended neither on age nor on treatment.

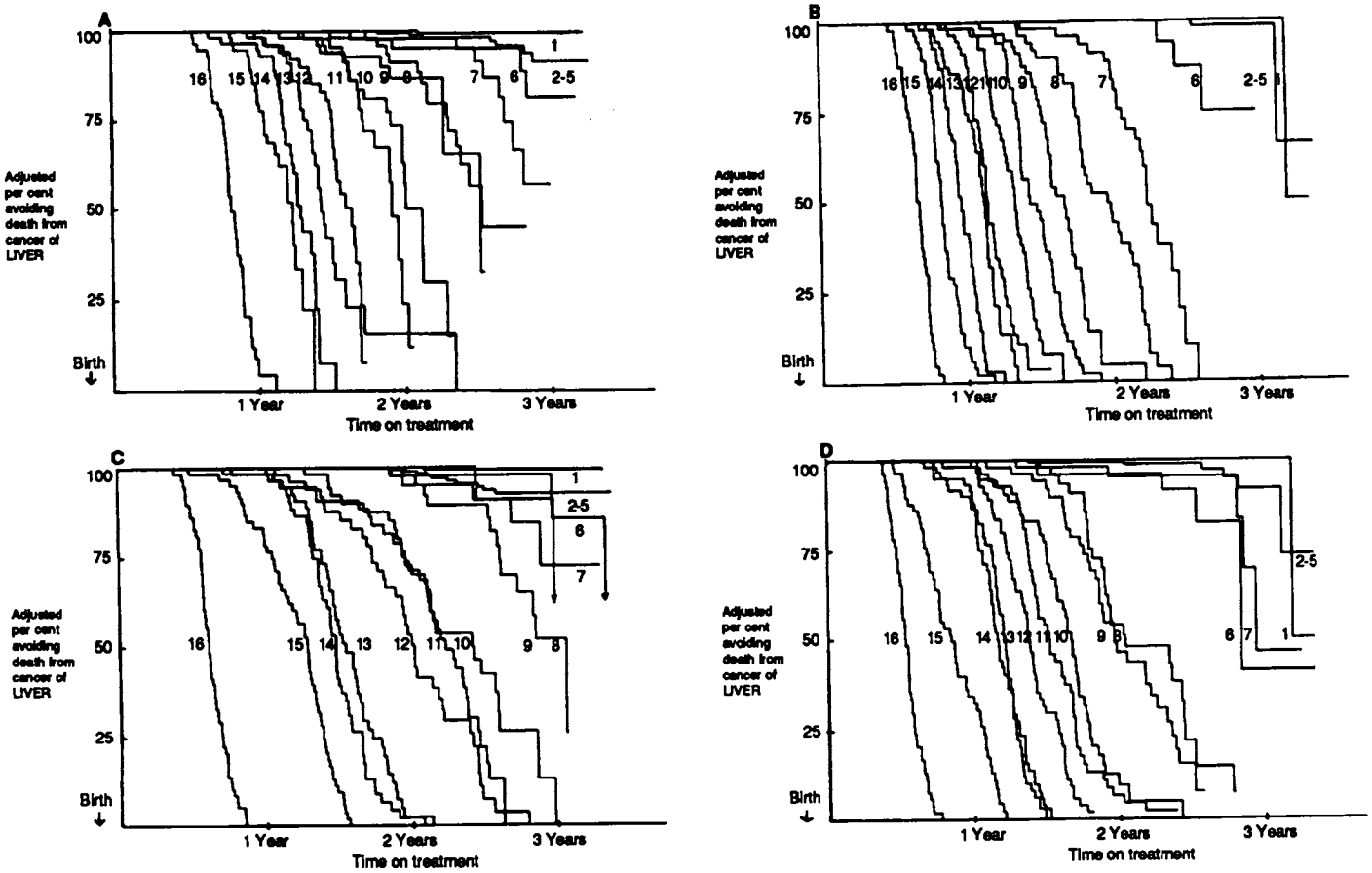


Fig. 6. Fatal liver cancer by dose level. *A*, NDEA, males; *B*, NDEA, females; *C*, NDMA, males; *D*, NDMA, females. For clarity, groups 2–5 are merged.

of incidental neoplasms only in the lower-dosed group (*e.g.*, 1–8).

3D. Use of "Contexts of Observation" (Fatal/Incidental). Both for fatal and for incidental lesions, the underlying statistical difficulty is that tumor onset rates among animals of a given age are generally much greater among old than among young animals. Consequently, if the onset rate of some particular type of tumor in a particular group of animals is to be characterized adequately, this cannot be achieved merely by specification of the crude percentage of tumor-bearing animals; instead, it essentially requires some way of describing the *dependence on age* of the cumulative tumor onset rate. Thus, two groups of animals may be said to be at similar risk with respect to some particular category of neoplasms if, and only if, the onset rates of such neoplasms *among animals of a given age* in each group are similar (irrespective of whether, just because one group died due to unrelated causes long before the other, the final percentages of animals with such neoplasms are quite dissimilar).

Unfortunately, one cannot observe directly exactly when an internal tumor first arises. One can, however, observe when it reaches a sufficient degree of severity to kill its host (or to cause the sacrifice of its host), and one can also observe the prevalence of internal tumors among animals that have died at various ages of unrelated causes. Consequently, it is possible to construct two graphs against age that summarize the experimental findings in a particular treatment group. One such graph is of the *prevalence* of incidental tumors at a particular anatomic site among the survivors at a given age, and the other is of the *cumulative mortality* by a given age from tumors at that site (or, equivalently, a "Kaplan-Meier" graph of the proportions of animals that would remain alive at each age if the death rates from such tumors were as observed and all other causes of death were prevented). Observations of incidental tumors can occasionally be used to construct "maximum likelihood" (3) graphs against age of the prevalence of such lesions, and observations of fatal tumors can frequently be used to construct "Kaplan-Meier" graphs against age characterizing the cumulative mortality from such lesions (3).[10]

Thus, two quite different statistical techniques had to be used to describe graphically the relationship with age of the prevalence of and the cumulative mortality from tumors of some particular type. Likewise, two quite different statistical techniques (the "prevalence" method and the "death rate" method) should be used to test whether treatment has any statistically significant effect on the pattern of dependence on age of *(a)* the prevalence of and *(b)* the death rate from one particular type of tumor.

For a particular anatomic site, a "prevalence" analysis involves comparing, for each treatment group, the numbers of animals observed to have incidental tumors with the numbers that would have been expected to have done so if treatment had

[10] Although it is in principle possible to multiply together these two graphs (to produce one graph against age of the proportion of animals that would be *both* alive *and* free of the disease of interest if all other causes of death were prevented), this may not be wise because generally (depending on whether the disease of interest is usually found in a fatal, or usually found in an incidental, context) one or the other of these two graphs will be based on too little data to be reliable, and it would not then be advisable to multiply a reliable graph by an unreliable one. (Indeed, even when there happen to be similar numbers of fatal and of incidental tumors, the shapes of the Kaplan-Meier graphs describing the fatal tumors appear to be more reliable than those of the maximum-likelihood graphs describing the incidental tumors, which is why more use is made of the former than of the latter.)

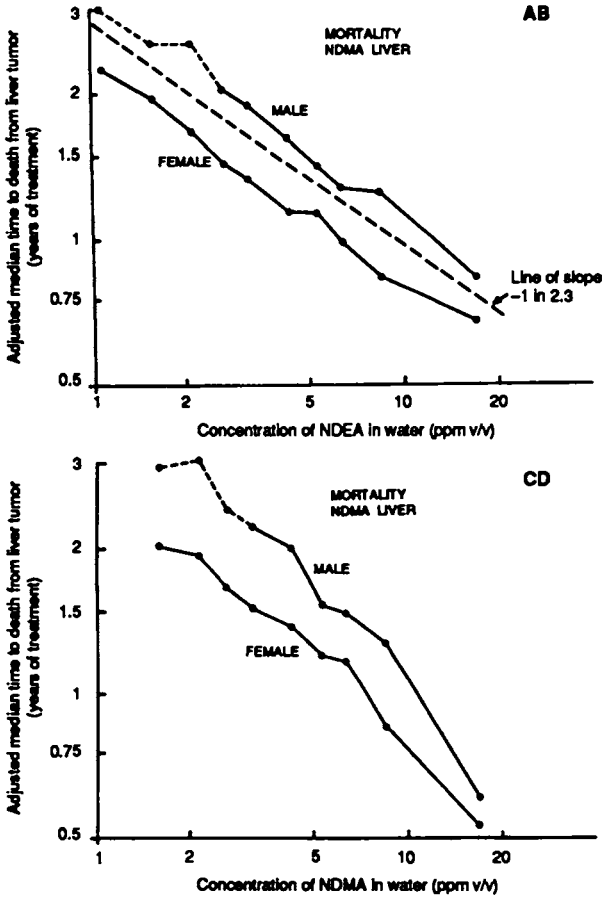


Fig. 7. *AB*, fatal liver neoplasms at high dose rates of NDEA. For histological details, see Tables 6A and 6B; in each sex, most were malignant liver cell hepatomas. Multiplication of ppm v/v by 9.23 yields μmol NDEA/liter. *CD*, fatal liver neoplasms at high dose rates of NDMA. For histological details, see Tables 6C and 6D; among males, most were malignant liver cell hepatomas, but among females most were benign bile duct tumors that caused death either directly or (by causing a palpable liver lesion) indirectly. These lines are composed of two segments with differing slopes; see Figs. 10–12. Multiplication of ppm v/v by 13.58 yields μmol NDMA/liter.

no effect.[11] For an example of such a tabulation, see Table 4, "Incidental Lesions."

A "death rate" analysis involves tabulating for each treatment group the numbers of animals observed to have died of the tumor type of interest with the numbers that would have been expected to have done so if, among animals of a given sex *who were still alive at a given age*, the risk of death in the near future from the tumor type of interest were the same as the risk seen in the aggregate of all of the treatment groups being compared.

Thus, the "at-risk" denominator for a *mortality* analysis should be the number of *survivors* at a given age, while the "at-risk" denominator for a *prevalence* analysis should be the number of *autopsies* following death at that age from unrelated causes. In an experiment such as the present one, where treatment strongly affects the mortality rate among animals of a given age, the ratio of the number of autopsies at a given age to the number of survivors at that age will vary considerably from group to group. Thus it is important to use the appropriate one as denominator.

Fortunately, however, what is generally wanted is an overall analysis of all tumor-bearing animals, irrespective of whether the tumors were "fatal" or "incidental" (because the distinction between these is chiefly needed just for arithmetic purposes and is usually of little biological interest). Consequently, separate analyses of fatal and of incidental neoplasms (as in Table 4) are generally added together to give an overall analysis (as in Table 3; the observed and expected numbers of Table 3 were actually derived, by such additions, from Table 4). Such addition eases the impossible requirement that all "contexts of observation" (3) be accurately determined, and replaces it by the more reasonable requirement that the autopsy procedures should be such as to minimize the number of individual errors that occur and to judge which tumors to call "fatal" and which "incidental" in such a way that the aggregate of the errors in each direction roughly counterbalance each other. In the present experiment, contexts of observation were classified as "definitely incidental," "probably incidental," "probably fatal," or "definitely fatal." No independent check of the validity of this classification exists, but it is reassuring to note that only 3% of the neoplasms fell into each of the two "probable" categories (the remainder being "definite") and that mortality described as being not "definitely" of "probably" *from* neoplasms of the liver or esophagus was unrelated to treatment (Fig. 2).

4. *Statistical Methods: Summary*

For each tumor type of interest, separate analyses are first performed of the observed and expected numbers of *(a)* fatal and *(b)* incidental such lesions. These two separate analyses are then combined to yield total observed and expected numbers of tumor-bearing animals. Although for the liver and esophagus both the separate and the combined analyses are discussed, for other sites it is the combined analysis that is chiefly of interest. For, although the distinction between fatal and incidental neoplasms is necessary to make unbiased correction for differences in longevity, it is a distinction that is (unlike distinctions based on tumor cell type, invasiveness, etc.) of little or no biological significance; indeed, it is obvious that any particular type of lesion that is found in a fatal context could have been found in an incidental context if its host had happened to die a few weeks earlier of unrelated causes.

Tests of statistical significance (which are, for tumors other than of the liver and esophagus, the principal aim of the present analysis) involve calculating T, a measure of the trend with respect to nitrosamine concentration of the $O - E$ values for each treatment group, z, the number of its standard errors by which T exceeds zero, and $1P$, an estimate of the one-tailed P value associated with z. The methods of estimation of $1P$ are described in "Appendix 1" and should be somewhat more reliable than a standard normal approximation.

5. *Autopsy Methods*

The chief objectives of the autopsy were: (*a*) the observation and sampling for histology of any macroscopic masses and, if any masses were observed in animals that had died naturally or that had been sacrificed because of sickness, (*b*) determination of which, if any, were thought likely to have been direct or indirect causes of death (or of the sickness that led to sacrifice). Thus, attention was concentrated on the macroscopic exami-

[11] That is, if among animals dying at about the same age (from causes other than the type of tumor that is being analyzed) the prevalence of incidental tumors in each separate group was the same as the prevalence in all groups together. The time periods used to define "about the same age" were derived from the flat parts of an ML prevalence graph for the aggregate of all animals of that sex in all the treatment groups being compared [as recommended by the International Agency for Research on Cancer (3)] excluding only animals with the congenital anomaly of a diaphragmatic hernia or animals in which the relevant tissue was autolyzed, cannibalized, or otherwise lost to autopsy.

Table 6A *Dose-response relationships for fatal liver neoplasms in control and NDEA-treated males*

Treatment group	NDEA concentration (ppm)	Animals dying of liver neoplasm arising from: Liver cells[b] (M)[c]	Bile ducts[d] (B)	Mesenchyme[e] (M)	Kupffer cells[f] (M)	Not known[g]	Total observed (*O*)	Total expected[a] (*E*)	Ratio (*O*/*E*)
1	0	1	0	0	0	0	1	61.4	0.02
2	0.033	0	0	1	0	0	1	15.6	0.06
3	0.066	0	0	0	0	0	0	16.4	0.00
4	0.132	0	0	4	1	0	5	16.1	0.31
5	0.264	0	0	1	0	1	2	14.5	0.14
6	0.528	3	0	1	0	0	4	16.2	0.25
7	1.056	5*	1	2	0	0	8	15.0	0.53
8	1.584	9	1*	2	1	1	14	14.1	0.99
9	2.112	6	0	1	0	0	7	11.2	0.62
10	2.640	15**	0	2	0	0	17	11.1	1.5
11	3.168	14	0	2	1	0	17	10.4	1.6
12	4.224	20	0	5	1	0	26	8.1	3.2
13	5.280	25	0	1	0	0	26	6.4	4.1
14	6.336	25	0	2	3	0	30	5.1	5.8
15	8.448	23	0	1	1	0	25	3.9	6.4
16	16.896	43	0	0	0	1	44	1.4	32.0
Total (all doses)		189	2	25	8	3	227	227.0	1.0

[a] Test for trend, any fatal liver neoplasms: $T = 1242$; $z = 39.79$.
[b] Test for trend, fatal hepatocellular neoplasms: $T = 1145$; $z = 37.98$.
[c] M, B, predominant histology for fatal neoplasms of this cell type: M, malignant; B, benign. The few exceptions to these cell type-specific predominant histologies are marked with asterisks, the number (*, **, etc.) of which corresponds to the number of such exceptions.
[d] Test for trend, fatal bile duct neoplasms: $T = 2$; $z = 3.25$.
[e] Test for trend, fatal mesenchymal neoplasms: $T = 50$; $z = 8.35$.
[f] Test for trend, fatal Kupffer cell neoplasms; $T = 30$; $z = 8.00$.
[g] Cell type and invasiveness not recorded due to autolysis or other loss of tissue.

Table 6B *Dose-response relationships for fatal liver neoplasms in control and NDEA-treated females*

Treatment group	NDEA concentration (ppm)	Animals dying of liver neoplasm arising from: Liver cells[b] (M)[c]	Bile ducts[d] (B)	Mesenchyme[e] (M)	Kupffer cells[f] (M)	Not known[g]	Total observed (*O*)	Total expected[a] (*E*)	Ratio (*O*/*E*)
1	0	0	1*	0	0	0	1	123.0	0.01
2	0.033	0	0	0	0	0	0	30.2	0.00
3	0.066	0	0	0	0	0	0	31.1	0.00
4	0.132	1	0	0	0	0	1	30.3	0.03
5	0.264	1	0	0	0	0	1	32.1	0.03
6	0.528	2	0	1	0	0	3	29.6	0.10
7	1.056	19*	2	1	1	0	23	29.6	0.78
8	1.584	35**	2	0	0	0	37	26.3	1.4
9	2.112	38****	0	0	0	0	38	24.6	1.5
10	2.640	45*	0	0	0	2	47	19.1	2.5
11	3.168	42**	0	0	0	0	42	16.4	2.6
12	4.224	41*	0	0	1	0	42	12.3	3.4
13	5.280	41	0	1	0	1	43	10.5	4.1
14	6.336	47**	0	0	0	0	47	7.4	6.4
15	8.448	55*	0	0	0	0	55	5.1	10.8
16	16.896	44*	0	2	0	3	49	1.4	34.7
Total (all doses)		411	5	5	2	6	429	429.0	1.0

[a] Test for trend, any fatal liver neoplasms: $T = 1937$; $z = 52.62$.
[b] Test for trend, fatal hepatocellular neoplasms: $T = 1847$; $z = 51.87$.
[c] M, B, predominant histology for fatal neoplasms of this cell type: M, malignant; B, benign. The few exceptions to these cell type-specific predominant histologies are marked with asterisks, the number (*, **, etc.) of which corresponds to the number of such exceptions.
[d] Test for trend, fatal bile duct neoplasms: $T = 4$; $z = 3.02$.
[e] Test for trend, fatal mesenchymal neoplasms: $T = 34$; $z = 6.10$.
[f] Test for trend, fatal Kupffer cell neoplasms: $T = 4$; $z = 4.03$.
[g] Cell type and invasiveness not recorded due to autolysis or other loss of tissue.

nation and description of most tissues, and although a sample was stored from any tissue found to be macroscopically abnormal, *routine* sampling and storage was confined to the liver (4 main lobes separately), esophagus, kidneys, bladder, lungs, and skull, which were taken from every intact animal. All tissues sampled were stored in 10% buffered formalin.

The liver was examined intact and the positions of all macroscopically evident lesions were recorded. Each lobe of the liver was cut into slices approximately 5 mm thick, and after macroscopic examination of the cut surfaces (and sectioning of any lesions seen there or elsewhere), two such slices of each lobe were fixed separately and stored, to facilitate further study of the correlation of the macroscopic findings at autopsy with any subsequent histological findings. One histological section of each of the four major lobes of the liver was then examined routinely for microscopic abnormalities.

The esophagus was removed, complete with the pharynx and base of the tongue, opened up to reveal the mucosal surface, and fixed intact on a stiff card with the serosal surface against the card. The general position and size of any macroscopically visible neoplasms or nodules were recorded and, unless they were extremely numerous, all were sectioned. In addition, one

Table 6C *Dose-response relationships for fatal liver neoplasms in control and NDMA-treated males*

Treatment group	NDMA concentration (ppm)	Animals dying of liver neoplasm arising from: Liver cells[b] (M)[c]	Bile ducts[d] (B)	Mesenchyme[e] (M)	Kupffer cells[f] (M)	Not known[g]	Total observed (*O*)	Total expected[a] (*E*)	Ratio (*O*/*E*)
1	0	1	0	0	0	0	1	86.3	0.01
2	0.033	1	0	0	0	0	1	23.0	0.04
3	0.066	1	1	0	1	0	3	22.4	0.13
4	0.132	1	1	1	0	0	3	22.0	0.14
5	0.264	1	1	1	0	0	3	21.9	0.14
6	0.528	2	1	0	0	0	3	23.9	0.13
7	1.056	2	0	1	2	0	5	21.0	0.24
8	1.584	3	0	0	0	0	3	21.1	0.14
9	2.112	4	3	6	0	0	13	21.7	0.60
10	2.640	11	7*	5	3	1	27	17.2	1.6
11	3.168	13*	8	12	0	0	33	17.1	1.9
12	4.224	18*	8*	10	0	0	36	14.6	2.5
13	5.280	27	14	3	2	0	46	10.4	4.4
14	6.336	30	12	7	0	0	49	9.4	5.2
15	8.448	44	7	4	0	0	55	6.4	8.6
16	16.896	46	0	10	0	3	59	1.7	34.5
Total (all doses)		205	63	60	8	4	340	340.0	1.0

[a] Test for trend, any fatal liver neoplasms: $T = 1900$; $z = 48.67$.
[b] Test for trend, fatal hepatocellular neoplasms: $T = 1330$; $z = 41.26$.
[c] M, B, predominant histology for fatal neoplasms of this cell type: M, malignant; B, benign. The few exceptions to these cell type-specific predominant histologies are marked with asterisks, the number (*, **, etc.) of which corresponds to the number of such exceptions.
[d] Test for trend, fatal bile duct neoplasms: $T = 216$; $z = 15.86$.
[e] Test for trend, fatal mesenchymal neoplasms: $T = 297$; $z = 19.63$.
[f] Test for trend, fatal Kupffer cell neoplasms: $T = 13$; $z = 3.33$
[g] Cell type and invasiveness not recorded due to autolysis or other loss of tissue.

Table 6D *Dose-response relationships for fatal liver neoplasms in control and NDMA-treated females*

Treatment group	NDMA concentration (ppm)	Animals dying of liver neoplasm arising from: Liver cells[b] (M)[c]	Bile ducts[d] (B)	Mesenchyme[e] (M)	Kupffer cells[f] (M)	Not known[g]	Total observed (*O*)	Total expected[a] (*E*)	Ratio (*O*/*E*)
1	0	0	1*	0	0	0	1	120.3	0.01
2	0.033	0	0	1	0	0	1	31.0	0.03
3	0.066	0	0	0	0	0	0	30.3	0.00
4	0.132	2*	0	0	0	0	2	28.8	0.07
5	0.264	1	1	1	0	0	3	31.3	0.10
6	0.528	3	0	1	1	0	5	29.2	0.17
7	1.056	2*	1	1	1	0	5	30.6	0.16
8	1.584	2	25	0	0	0	27	25.5	1.1
9	2.112	6	23	3	1	0	33	25.4	1.3
10	2.640	5*	38	1	0	0	44	21.2	2.1
11	3.168	4	41	3	0	0	48	19.4	2.5
12	4.224	7	43	2	0	1	53	16.0	3.3
13	5.280	12	39	0	0	1	52	11.2	4.6
14	6.336	19	30	2	0	0	51	10.5	4.9
15	8.448	39	9	5	0	2	55	5.5	10.0
16	16.896	41	1	12	0	4	58	1.9	30.8
Total (all doses)		143	252	32	3	8	438	438.0	1.0

[a] Test for trend, any fatal liver neoplasms: $T = 2110$; $z = 52.15$.
[b] Test for trend, fatal hepatocellular neoplasms: $T = 1047$; $z = 36.18$.
[c] M, B, predominant histology for fatal neoplasms of this cell type: M, malignant; B, benign. The few exceptions to these cell type-specific predominant histologies are marked with asterisks, the number (*, **, etc.) of which corresponds to the number of such exceptions.
[d] Test for trend, fatal bile duct neoplasms: $T = 751$; $z = 34.01$.
[e] Test for trend, fatal mesenchymal neoplasms: $T = 236$; $z = 16.00$.
[f] Test for trend, fatal Kupffer cell neoplasms: $T = 2$; $z = 0.91$.
[g] Cell type and invasiveness not recorded due to autolysis or other loss of tissue.

histological section from what remained of the esophagus was examined routinely for microscopic abnormalities.

The bladder was distended with formalin before removal to ensure fixation of the epithelium. The lungs were also inflated *in situ* with formalin through the trachea and then removed and fixed intact. The skull was fixed after removal of the brain and pituitary, and examination of the buccal cavity was effected by cutting through one axis of the jaw. The spinal cord was examined *in situ* by trimming off the dorsal parts of the neural arches with bone nibblers.

No formal mechanisms existed to ensure that the person undertaking an autopsy was "blind" to the treatment that the dead animal had received, and indeed, since about one-third of the animals had large neoplasms of the liver or esophagus that were known to have been caused by nitrosamines, such a "blind" procedure would have been difficult to devise. At autopsy, lack of blindness to treatment will not affect the detection of large, fatal neoplasms, but it might in principle affect either the probability of macroscopic detection of small, incidental neoplasms or the probability that a lesion of doubtful lethality will be classified as having been fatal. The staff concerned with the autopsies, however, consider it improbable that either bias

Table 7A *Dose-response relationships for incidental liver neoplasms in control and NDEA-treated males that were not thought to have died of any liver neoplasm, and whose livers came to autopsy without significant autolysis, cannibalism, or diaphragmatic hernia*

Treatment group	NDEA concentration (ppm)	No. of such rats	No. with incidental malignant neoplasms of							No. with any incidental neoplasm of						
			Liver cells	Bile ducts	Mesenchyme	Kupffer cells	Any part (*O*)	Expected (*E*)	Ratio (*O*/*E*)	Liver cells	Bile ducts	Mesenchyme	Kupffer cells	Any part (*O*)	Expected (*E*)	Ratio (*O*/*E*)
1	0	231	3	0	0	0	3	13.89	0.22	9	3	0	0	12	24.74	0.49
2	0.033	59	0	0	0	0	0	3.49	0.00	1	2	0	0	3	5.95	0.50
3	0.066	60	0	0	0	0	0	3.56	0.00	2	0	0	0	2	6.21	0.32
4	0.132	55	1	0	0	0	1	3.45	0.29	3	1	0	0	4	6.14	0.65
5	0.264	57	0	0	0	0	0	3.38	0.00	0	2	0	0	2	5.93	0.34
6	0.528	56	2	0	2	0	3	3.21	0.93	2	0	2	0	3	5.58	0.54
7	1.056	52	3	1	0	0	4	2.92	1.37	4	3	1	0	7	4.97	1.41
8	1.584	46	7	0	1	0	7	2.59	2.70	9	2	1	0	11	4.35	2.53
9	2.112	51	2	0	2	0	4	2.85	1.40	4	1	3	0	8	4.78	1.67
10	2.640	43	5	0	0	0	5	2.37	2.11	6	2	0	0	8	3.95	2.02
11	3.168	43	6	0	2	0	8	2.54	3.15	6	2	2	0	9	4.25	2.12
12	4.224	34	1	0	1	0	2	1.99	1.01	3	2	1	0	5	3.25	1.54
13	5.280	34	2	0	0	0	2	2.03	0.98	2	1	0	0	3	3.28	0.91
14	6.336	29	3	0	1	1	5	1.76	2.85	3	0	1	1	5	2.80	1.79
15	8.448	35	5	0	0	0	5	2.21	2.26	5	0	0	0	5	3.57	1.40
16	16.896	16	4	0	0	0	4	0.76	5.27	4	0	0	0	4	1.26	3.18
Total (all doses)		901	44	1	9	1	53	53.00	1.00	63	21	11	1	91	91.00	1.00

Table 7B *Dose-response relationships for incidental liver neoplasms in control and NDEA-treated females that were not thought to have died of any liver neoplasm, and whose livers came to autopsy without significant autolysis, cannibalism, or diaphragmatic hernia*

Treatment group	NDEA concentration (ppm)	No. of such rats	No. with incidental malignant neoplasm of							No. with any incidental neoplasm of						
			Liver cells	Bile ducts	Mesenchyme	Kupffer cells	Any part (*O*)	Expected (*E*)	Ratio (*O*/*E*)	Liver cells	Bile ducts	Mesenchyme	Kupffer cells	Any part (*O*)	Expected (*E*)	Ratio (*O*/*E*)
1	0	234	2	0	0	1	3	15.94	0.19	11	3	0	1	15	36.86	0.41
2	0.033	55	0	0	0	0	0	3.73	0.00	4	2	0	0	6	8.86	0.68
3	0.066	57	2	0	0	0	2	3.96	0.50	4	0	0	0	4	8.62	0.46
4	0.132	59	0	0	0	0	0	3.70	0.00	2	1	0	0	3	8.13	0.37
5	0.264	57	2	0	0	1	3	4.02	0.75	3	2	0	1	6	9.83	0.61
6	0.528	55	1	0	0	0	1	3.36	0.30	8	1	0	0	9	7.20	1.25
7	1.056	37	4	0	0	0	4	2.40	1.66	12	0	0	0	12	4.25	2.82
8	1.584	22	6	0	0	0	6	1.63	3.67	7	0	0	0	7	2.52	2.78
9	2.112	27	3	0	0	0	3	1.82	1.65	7	2	0	0	9	3.10	2.90
10	2.640	13	0	0	0	0	0	0.98	0.00	0	0	0	0	0	1.45	0.00
11	3.168	18	3	0	1	0	4	1.23	3.26	3	0	1	0	4	2.03	1.97
12	4.224	18	6	0	0	0	6	1.17	5.14	7	0	0	0	7	2.03	3.44
13	5.280	17	6	0	0	0	6	1.00	5.99	9	1	0	0	9	1.93	4.65
14	6.336	12	5	0	0	0	5	0.70	7.14	5	0	0	0	5	1.37	3.66
15	8.448	4	2	0	0	0	2	0.19	10.30	2	0	0	0	2	0.46	4.34
16	16.896	4	1	0	0	0	1	0.15	6.87	1	0	0	0	1	0.35	2.90
Total (all doses)		689	43	0	1	2	46	46.00	1.00	85	12	1	2	99	99.00	1.00

Table 7C *Dose-response relationships for incidental liver neoplasms in control and NDMA-treated males that were not thought to have died of any liver neoplasm, and whose livers came to autopsy without significant autolysis, cannibalism, or diaphragmatic hernia*

Treatment group	NDMA concentration (ppm)	No. of such rats	No. with incidental malignant neoplasm of							No. with any incidental neoplasm of						
			Liver cells	Bile ducts	Mesenchyme	Kupffer cells	Any part (*O*)	Expected (*E*)	Ratio (*O*/*E*)	Liver cells	Bile ducts	Mesenchyme	Kupffer cells	Any part (*O*)	Expected (*E*)	Ratio (*O*/*E*)
1	0	231	3	0	0	0	3	6.40	0.47	9	3	0	0	12	23.34	0.51
2	0.033	58	0	0	0	0	0	1.69	0.00	3	2	0	0	4	6.22	0.64
3	0.066	56	1	0	0	0	1	1.55	0.65	2	2	0	0	4	5.75	0.70
4	0.132	56	0	0	0	0	0	1.58	0.00	1	1	0	0	2	5.72	0.35
5	0.264	56	0	0	0	0	0	1.36	0.00	3	1	0	0	3	5.82	0.52
6	0.528	57	1	0	0	0	1	1.56	0.64	2	0	0	0	2	5.93	0.34
7	1.056	54	3	0	0	0	3	1.50	2.00	3	1	0	0	4	5.27	0.76
8	1.584	56	3	0	0	0	3	1.19	2.53	5	4	0	0	9	4.92	1.83
9	2.112	47	2	0	0	0	2	0.97	2.06	3	4	1	0	6	4.09	1.47
10	2.640	32	2	0	0	0	2	0.47	4.27	2	6	0	0	8	2.08	3.84
11	3.168	27	1	0	0	0	1	0.38	2.65	1	4	0	0	5	1.97	2.53
12	4.224	24	1	0	0	0	1	0.13	7.81	1	4	0	0	5	1.11	4.52
13	5.280	14	0	0	0	0	0	0.11	0.00	0	2	0	0	2	0.96	2.07
14	6.336	9	2	0	0	0	2	0.07	27.01	2	6	0	0	7	0.55	12.71
15	8.448	4	0	0	0	0	0	0.04	0.00	0	1	0	0	1	0.27	3.71
16	16.896	1	0	0	0	0	0	0.00	0.00	0	0	0	0	0	0.00	0.00
Total (all doses)		782	19	0	0	0	19	19.00	1.00	37	41	1	0	74	74.00	1.00

Table 7D *Dose-response relationships for incidental liver neoplasms in control and NDMA-treated females that were not thought to have died of any liver neoplasm, and whose livers came to autopsy without significant autolysis, cannibalism, or diaphragmatic hernia*

Treatment group	NDMA concentration (ppm)	No. of such rats	No. with incidental malignant neoplasm of							No. with any incidental neoplasm of						
			Liver cells	Bile ducts	Mesenchyme	Kupffer cells	Any part (*O*)	Expected (*E*)	Ratio (*O*/*E*)	Liver cells	Bile ducts	Mesenchyme	Kupffer cells	Any part (*O*)	Expected (*E*)	Ratio (*O*/*E*)
1	0	234	2	0	0	1	3	4.29	0.70	11	3	0	1	15	37.57	0.40
2	0.033	58	0	0	0	0	0	1.15	0.00	2	1	0	0	3	9.98	0.30
3	0.066	59	0	0	0	0	0	1.25	0.00	2	4	0	0	6	10.17	0.59
4	0.132	56	1	0	0	0	1	0.87	1.15	2	1	0	0	3	8.77	0.34
5	0.264	54	0	0	0	0	0	1.09	0.00	1	3	0	0	4	9.08	0.44
6	0.528	55	2	0	0	0	2	0.87	2.30	3	4	0	0	7	8.20	0.85
7	1.056	54	2	0	1	0	3	1.10	2.72	4	8	1	0	13	9.05	1.44
8	1.584	30	0	0	0	0	0	0.31	0.00	1	14	0	0	15	4.57	3.28
9	2.112	26	0	0	0	0	0	0.28	0.00	1	10	0	0	10	3.84	2.60
10	2.640	16	0	0	0	0	0	0.18	0.00	2	6	0	0	7	2.27	3.09
11	3.168	12	0	0	0	0	0	0.17	0.00	0	7	0	0	7	1.77	3.95
12	4.224	7	0	0	0	0	0	0.11	0.00	0	3	0	0	3	0.94	3.18
13	5.280	8	1	0	0	0	1	0.13	7.83	1	5	0	0	6	1.06	5.65
14	6.336	9	1	0	0	0	1	0.15	6.77	1	8	0	0	8	1.28	6.25
15	8.448	5	1	0	0	0	1	0.06	16.87	1	1	0	0	2	0.46	4.36
16	16.896	1	0	0	0	0	0	0.00	0.00	0	0	0	0	0	0.00	0.00
Total (all doses)		684	10	0	1	1	12	12.00	1.00	32	78	1	1	109	109.00	1.00

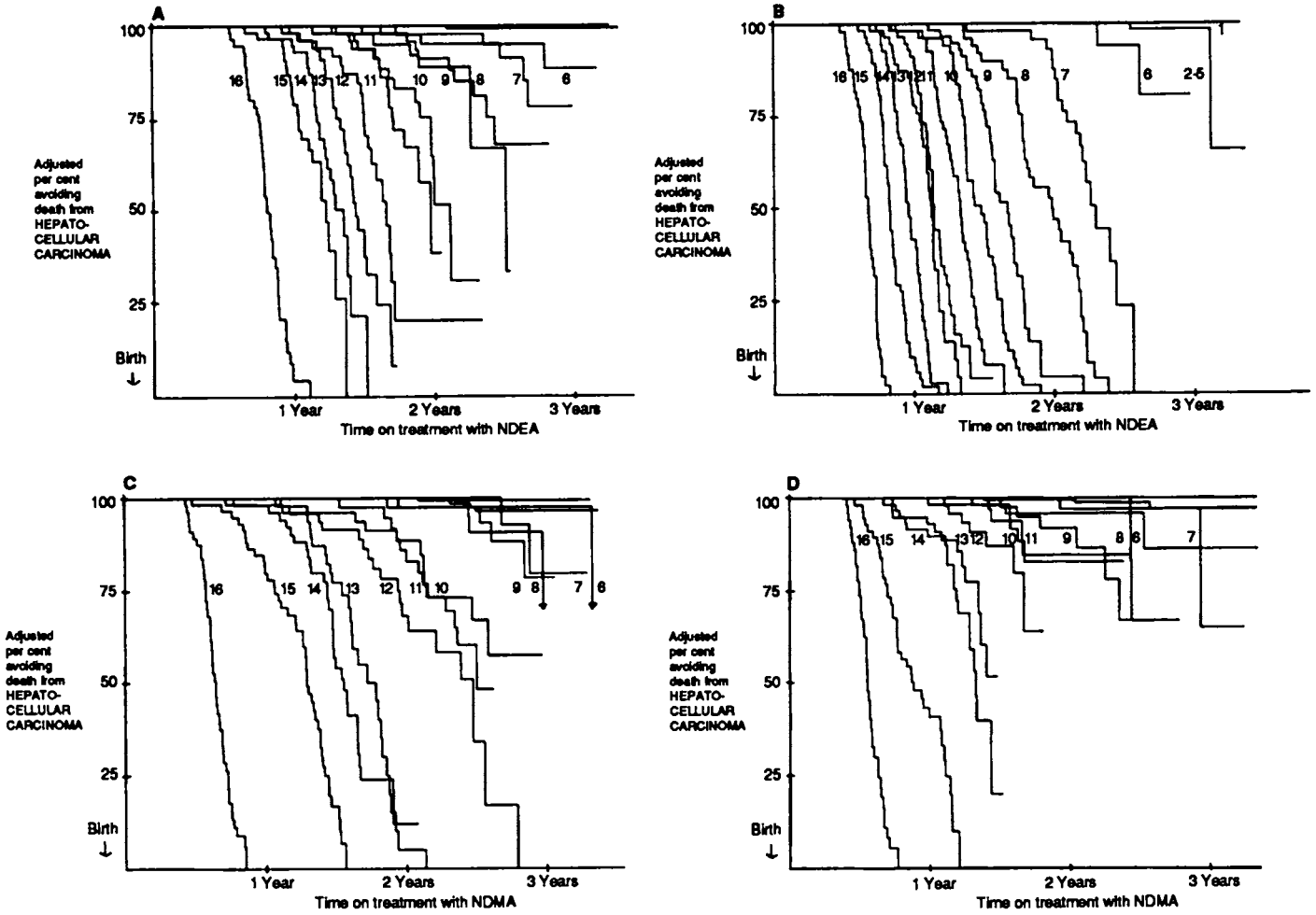


Fig. 8. Fatal hepatocellular carcinoma by dose level. *A*, NDEA, males; *B*, NDEA, females; *C*, NDMA, males; *D*, NDMA, females.

materially affected their reported findings, and certainly the lack of apparent relationship to treatment of the aggregate of all neoplasms other than of the liver and esophagus (see "Results") tends to support their view, at least for such neoplasms. For neoplasms of the liver and esophagus, assessment of any biases due to nonblind autopsy procedures is more difficult. In animals with no gross neoplasms in the esophagus or with no gross neoplasms in the liver it is impossible to prove that knowledge of treatment did not affect the care with which small neoplasms were sought in those organs. However, the firm opinion of the staff that no material bias was introduced is reassuring, especially since they appear to have been correct

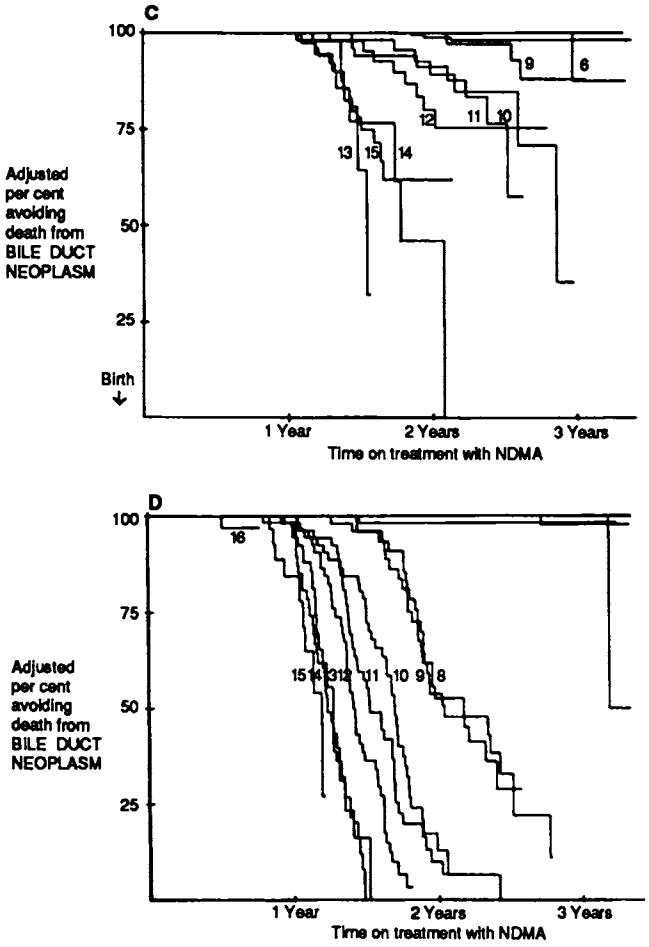


Fig. 9. Fatal bile duct neoplasm by NDMA dose level. (Too few such neoplasms were induced by NDEA for *A* and *B* to be plotted.) *C*, males; *D*, females.

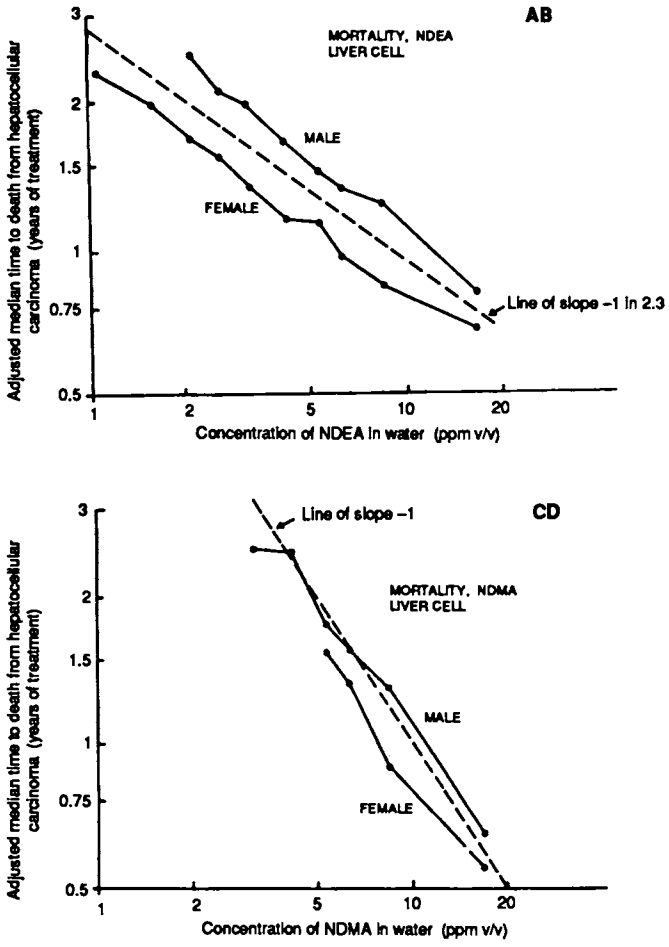


Fig. 10. *AB*, fatal hepatocellular carcinomas at high dose rates of NDEA. Multiplication of ppm v/v by 9.23 yields μmol NDEA/liter. *CD*, fatal hepatocellular carcinomas at high dose rates of NDMA. Multiplication of ppm v/v by 13.58 yields μmol NDMA/liter.

about the lack of substantial bias at other sites. [Note that in animals with a gross neoplasm in the esophagus or with a gross neoplasm in the liver, any bias in the assessment of what other lesions were macroscopically detectable in the affected organ(s) is of little relevance, since the statistical analyses relate chiefly to whether or not *any* neoplasms were present in an organ, not to how many were present.]

6. *Histological Methods*

All macroscopically abnormal tissues collected at autopsy were examined after fixation and, with reference to the autopsy report on where macroscopic masses were observed, appropriate samples were trimmed. All samples were processed with an automated wax-embedding technique and stained with hematoxylin and eosin. Microscopic examination of the sections was then carried out, chiefly to identify the histological nature of any macroscopic lesions seen at autopsy. In addition to the examination of macroscopically visible lesions, four sections from the liver and one from the esophagus were routinely examined for the presence of various microscopic abnormalities.

Where tumors or other lesions were seen, each was identified according to the probable cell type of origin.[12] In addition, for solid tumors, the degree of differentiation, the presence or

[12] "Mesenchymal" liver tumors were, however, not further subdivided into hemangio-sarcomas, -pericytomas, and -endotheliomas, and these three types are therefore viewed together.

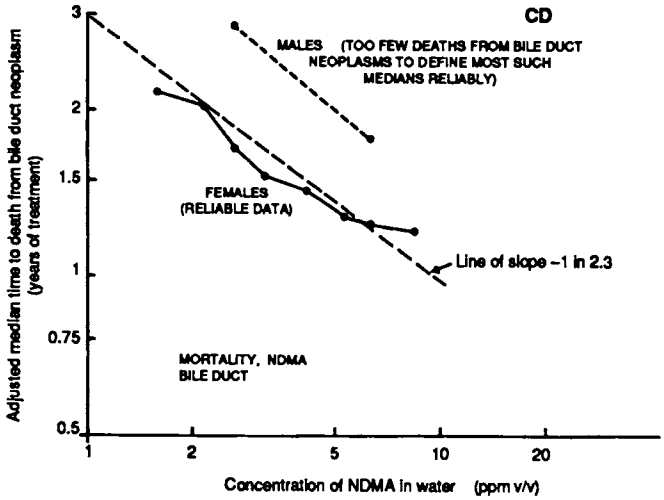


Fig. 11. *CD*, fatal bile duct neoplasms at high dose rates of DMN. Although at 1 ppm almost no females died of bile duct neoplasms, this probably reflects the play of chance rather than a true threshold, for several *non*-fatal such neoplasms arose; see Tables 6D and 7D. Also, multiplication of ppm v/v by 13.58 yields μmol NDMA/liter.

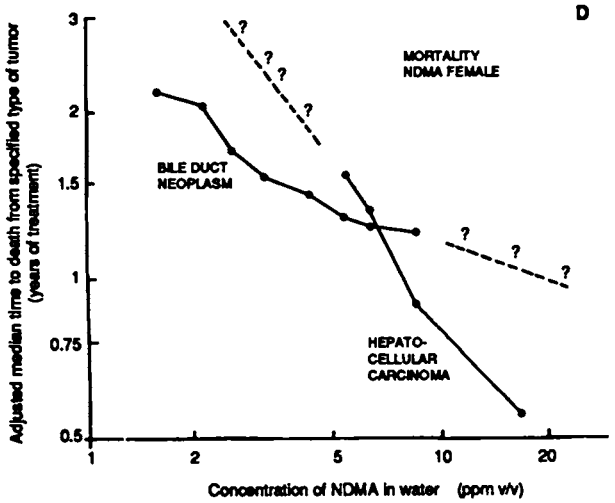


Fig. 12. *D*, contrasting dose-response relationships for bile duct neoplasms (*shallow slope*, at least above 3 ppm), and hepatocellular carcinomas (*steep slope*) among NDMA-treated females. Combination of this shallow with this steep dose-response relationship implies that the overall dose-response relationship for mortality from total liver neoplasms (all cell types) must change slope at a concentration of about 5 ppm, being steeper at high doses (where hepatocellular carcinomas predominate) than at intermediate doses (where bile duct neoplasms predominate. Multiplication of ppm v/v by 13.58 yields μmol NDMA/liter.

absence of invasion or metastasis, and the general appearance of the cells were noted. Finally, using these observations, each solid tumor that was examined microscopically was classified as being benign or malignant, and for most types of tumor the malignancy was graded as low, medium, or high. In the opinion of the one histologist (Dr. P. Grasso) who examined all these slides, however, the criteria by which the latter distinctions were made may well have fluctuated more widely than did the more consistent criteria distinguishing between malignant, benign, and nonneoplastic. Consequently, the analyses of the lesions at particular anatomic sites have been either of "all tumors (benign or malignant)" or of "malignant tumors," in neither case attempting any finer subcategorization of the malignancy.

Rereading of the slides would undoubtedly lead to some "benign neoplasms" as nonneoplastic or as malignant lesions and *vice versa*, with the degree of reclassification to be anticipated being perhaps moderately substantial even if the same histologist undertook the review, especially since a few years have now passed. Nevertheless, the original histology was done in ignorance of the treatment each rat had received (except, when examining sections from an esophagus or liver, insofar as lesions in them made this obvious). There is thus little likelihood of any material treatment-related bias being introduced by the inevitable random errors in the histological distinction among malignant, benign, and other lesions (except, perhaps, in the generally irrelevant detection of extra neoplastic lesions in the liver or esophagus of a rat which already has one or more such lesions clearly evident).

7. *Palpation Methods*

The one feature of the conduction of the experiment where some treatment-related bias undoubtedly arose, at least in the early months of the study, was the weekly palpation. The plan was for animals to be palpated weekly and sacrificed if palpable liver lesions were present, in the hope of monitoring the onset of malignancies at a stage well before they were likely to cause death. Unfortunately, not only did 28 animals (of 4080) develop palpable cysts or nodules that led to their premature sacrifice before any malignant disease was macroscopically evident, but also 27 animals were sacrificed in error, with livers that appeared normal at autopsy. These errors occurred chiefly during the early months of the study, after which the palpation criteria for sacrifice became more strict. What is disturbing about the early palpation errors, however, is that (presumably because the staff responsible for palpation were not "blind" to treatment) they were more common in the high- than in the low-dosed groups. This bias may have affected only the early months, before much experience had been gained, in which case it would have little or no effect on the characterization of the dose-response relationship among the low-dosed animals (*e.g.*, in groups 1–8).

8. *Units of Dosage*

The doses were originally specified in ppm (v/v) in the drinking water. These doses can be multiplied by the densities of NDEA and NDMA [0.9422 and 1.0061, respectively (1)] to yield ppm (w/w). Further division by the molecular weights of NDEA and NDMA [102.1 and 74.1, respectively (1)] yields mmol/liter. Thus, overall, multiplication of the basic ppm (v/v, as used throughout the present text) by 9.23 (NDEA) or by 13.58 (NDMA) will yield micromolar concentrations, which may be more appropriate for purposes of comparison of the quantitative effects of the two nitrosamines on the liver.

Both water intake and body weight varied with age (see Appendix Figs. A, B, and C), although not in direct proportion to each other. Hence, so did their ratio (ml/kg/day). Consequently, a particular concentration expressed in ppm or μmol in the drinking water implies a dose in mg/kg/day that varies with age (although between the 6th and the 24th month of treatment this variation was not large). Moreover, there are inevitably errors of measurement in both body weight and, especially, water intake (although only a few % of the water was thought to have been wasted). If, despite this, approximate conversion to mg/kg/day is required, this may be achieved by noting that recorded water intake was not materially affected by treatment and that at 1 year of age it averaged about 72 ml/kg/day for females and 41 ml/kg/day for males. However, concentration in the drinking water is biologically about as satisfactory a way of expressing dose as mg/kg/day; thus all analyses and tests for trends that follow will be with respect to dose in the original, reliably known, units of ppm (v/v).

Results

When separate analyses are needed of the effects of A: NDEA on males, B:NDEA on females, C:NDMA on males, and D:NDMA on females, the corresponding table numbers and figure numbers will be subscripted A, B, C, or D accordingly (or AB for NDEA in both sexes and CD for NDMA in both sexes).[13]

The results are subdivided into a few main sections: Section 1, descriptions of the onset rates of tumors of the *esophagus* or

[13] With regard to the labeling of figures, some figures will appear to be missing sections; *e.g.*, there are Figs. 11*CD* and 12*D* but no Figs. 11*AB*, 12*AB*, or 12*C*. This occurrence is a factor of the nature of the data. For example, since there were only 8 bile duct neoplasms in NDEA-treated rats, no dose response is possible; hence, there are no Figs. 11*AB* or 12*AB*. Bile duct neoplasms were also less common among NDMA-exposed males ($n = 63$) than among females ($n = 252$) (Tables 6 and 7), and so the male dose-response data are unreliable. Hence, there is no Fig. 12*C*.

Table 8A *Eight types of nonneoplastic liver lesions in control and NDEA-treated male rats that are strongly related to the treatment: numbers of affected animals and tests for trend with respect to dose*

Treatment group	NDEA concentration (ppm)	No. of animals at risk[a]	No. of at-risk animals with: Hyperplastic nodules	Bile duct hyperplasia	Kupffer cell hyperplasia	Cytomegaly	Shrinkage of hepatocytes	Abnormality of glycogen-containing cells	Cysts[b]	Any of these lesions: Observed (*O*)	Expected (*E*)	Ratio (*O*/*E*)
1	0	219	4	3	0	4	42	17	24	82	112.1	0.7
2	0.033	56	2	0	0	1	8	4	6	18	28.4	0.6
3	0.066	58	2	1	0	1	10	0	8	18	30.1	0.6
4	0.132	51	3	0	0	0	13	0	7	20	26.1	0.8
5	0.264	55	1	0	0	1	24	0	8	30	26.4	1.1
6	0.528	53	4	2	0	0	23	1	16	33	27.8	1.2
7	1.056	45	6	0	0	0	19	4	10	30	20.8	1.4
8	1.584	35	6	2	0	4	15	7	8	26	16.3	1.6
9	2.112	43	4	0	0	0	9	7	7	20	17.2	1.2
10	2.640	35	7	0	0	1	8	7	2	18	14.2	1.3
11	3.168	34	5	0	0	3	8	5	6	18	12.8	1.4
12	4.224	29	2	2	0	1	6	8	0	15	10.8	1.4
13	5.280	31	5	1	0	2	3	3	1	13	10.8	1.2
14	6.336	24	3	0	0	1	2	5	5	12	8.7	1.4
15	8.448	30	5	3	0	1	2	8	4	16	10.7	1.5
16	16.896	12	2	0	1	1	3	3	3	8	4.0	2.0
Total (all doses)		810	61	14	1	21	195	79	115	377	377.0	1.0
Tests for positive trend:[d] 1*P* =			[e]	0.4%	0.1%	[e]	[e]	[e]	[e]	[e]		

[a] Number of rats whose livers came to full autopsy with no neoplasm(s), no diaphragmatic hernia, and no significant autolysis, cannibalism, or other loss of tissues to autopsy.
[b] Cysts, excluding any that were clearly due to lesions originating in the bile ducts. (Many of the remaining cysts were due to preneoplastic lesions of the blood vessels and thus contained some blood.)
[c] *O*/*E* values among the higher-dosed groups may suggest a misleadingly shallow dose-response relationship; see text.
[d] For statistical methods, see text or Ref. 3. "Death rate" methods were used for that minority of lesions which, by causing the liver to become palpable, led to sacrifice. "Prevalence methods" were used for the remainder, and the two analyses were combined in the usual way. The 8 pairs of *T* and *z* values that this generated were: 100, 5.8; 24, 3.1; 16, 7.0; 39, 5.1; 86, 5.0; 137, 7.4; 91, 6.1; and 222, 7.2.
[e] $1P < 0.05\%$.

Table 8B *Eight types of nonneoplastic liver lesions in control and NDEA-treated female rats that are strongly related to treatment: numbers of affected animals and tests for trend with respect to dose*

Treatment group	NDEA concentration (ppm)	No. of animals at risk[a]	No. of at-risk animals with: Hyperplastic nodules	Bile duct hyperplasia	Kupffer cell hyperplasia	Cytomegaly	Shrinkage of hepatocytes	Abnormality of glycogen-containing cells	Cysts[b]	Any of these lesions: Observed (*O*)	Expected (*E*)	Ratio (*O*/*E*)
1	0	219	14	6	0	7	36	11	7	64	96.1	0.7
2	0.033	49	3	0	0	0	7	0	2	11	22.9	0.5
3	0.066	53	6	0	0	0	11	0	4	18	23.7	0.8
4	0.132	56	1	0	0	1	14	2	2	20	24.6	0.8
5	0.264	51	9	0	1	0	16	2	5	27	23.4	1.2
6	0.528	46	8	0	0	2	9	2	3	21	18.5	1.1
7	1.056	25	6	0	0	1	5	5	2	16	10.9	1.5
8	1.584	15	8	1	0	2	1	2	4	9	6.6	1.4
9	2.112	18	10	0	0	6	0	3	2	13	7.8	1.7
10	2.640	13	8	0	0	4	3	8	1	12	4.6	2.6
11	3.168	14	10	1	1	3	3	4	4	13	4.0	3.3
12	4.224	11	9	0	0	4	1	3	1	10	2.5	4.0
13	5.280	8	5	0	0	1	1	2	0	6	1.7	3.5
14	6.336	7	4	0	0	3	0	2	1	6	1.7	3.6
15	8.448	2	0	0	0	1	0	1	0	2	0.6	3.6
16	16.896	3	1	0	0	1	1	0	0	2	0.5	4.0
Total (all doses)		590	102	8	2	36	108	47	38	250	250.0	1.0
Tests for positive trend:[d] 1*P* =			[e]	8.3%	4.4%	[e]	[e]	[e]	[e]	[e]		

[a] Number of rats whose livers came to full autopsy with no neoplasm(s), no diaphragmatic hernia, and no significant autolysis, cannibalism, or other loss of tissues to autopsy.
[b] Cysts, excluding any that were clearly due to lesions originating in the bile ducts. (Many of the remaining cysts were due to preneoplastic lesions of the blood vessels and thus contained some blood.)
[c] *O*/*E* values among the higher-dosed groups may suggest a misleadingly shallow dose-response relationship; see text.
[d] For statistical methods, see text or Ref. 3. "Death rate" methods were used for that minority of lesions which, by causing the liver to become palpable, led to sacrifice. "Prevalence methods" were used for the remainder, and the two analyses were combined in the usual way. The 8 pairs of *T* and *z* values that this generated were: 146, 11.8; 2, 1.5; 2, 2.2; 81, 10.3; 33, 5.6; 61, 7.6; 20, 6.2; and 189, 11.5.
[e] $1P \leq 0.05\%$.

liver at dose rates (*e.g.*, more than 1–2 ppm) so high that the dose-response relationship would be uninterpretable without proper statistical allowance for differences in longevity on the tumor yields in each group. At these *high* dose rates, the median time to death just from the neoplasm of interest can be estimated, and the emphasis will chiefly be on tumors that were observed in a *fatal*, rather than an incidental, context; Section 2, brief details of statistical significance of any trends with respect to dose rate of the numbers of animals with tumors at various sites (or groups of sites) other than the liver or esoph-

Table 8C *Eight types of nonneoplastic liver lesions in control and NDMA-treated male rats that are strongly related to treatment: numbers of affected animals and tests for trend with respect to dose*

Treatment group	NDMA concentration (ppm)	No. of animals at risk[a]	No. of at-risk animals with: Hyperplastic nodules	Bile duct hyperplasia	Kupffer cell hyperplasia	Cytomegaly	Shrinkage of hepatocytes	Abnormality of glycogen-containing cells	Cysts[b]	Any of these lesions: Observed (O)	Expected (E)	Ratio (O/E)
1	0	219	4	3		4	42	17	24	82	100.1	0.8
2	0.033	54	4	0		0	10	1	6	18	25.1	0.7
3	0.066	52	4	0		1	9	1	7	17	23.8	0.7
4	0.132	54	1	0		2	12	0	4	14	24.8	0.6
5	0.264	53	1	1		1	10	3	10	22	23.5	0.9
6	0.528	55	3	0		1	13	0	8	22	25.9	0.8
7	1.056	50	3	0		2	18	1	15	28	21.1	1.3
8	1.584	47	3	1		5	17	3	12	29	18.6	1.6
9	2.112	41	6	0		4	12	2	6	21	16.7	1.3
10	2.640	24	4	1		2	3	4	3	12	7.4	1.6
11	3.168	22	5	2		3	6	5	5	16	7.0	2.3
12	4.224	19	3	0		1	3	1	5	10	4.1	2.5
13	5.280	12	3	0		2	4	2	2	7	3.6	2.0
14	6.336	2	0	0		0	0	1	0	1	0.4	2.5
15	8.448	3	0	0		2	2	2	2	3	1.0	2.9
16	16.896	1	0	0		1	0	0	0	1	0.1	16.0
Total (all doses)		708	44	8	0	31	161	43	109	303	303.0	1.0
Tests for positive trend:[d] 1P =			[e]	1.5%		[e]	[e]	0.9%	[e]	[e]		

[a] Numbers of rats whose livers came to full autopsy with no neoplasm(s), no diaphragmatic hernia, and no significant autolysis, cannibalism, or other loss of tissues to autopsy.
[b] Cysts, excluding any that were clearly due to lesions originating in the bile ducts. (Many of the remaining cysts were due to preneoplastic lesions of the blood vessels and thus contained some blood.)
[c] *O/E* values among the higher-dosed groups may suggest a misleadingly shallow dose-response relationship; see text.
[d] For statistical methods, see text or Ref. 3. "Death rate" methods were used for that minority of lesions which, by causing the liver to become palpable, led to sacrifice. "Prevalence methods" were used for the remainder, and the two analyses were combined in the usual way. The 8 pairs of T and z values that this generated were: 49, 7.6; 6, 2.5; –, –; 59, 9.0; 70, 6.7; 25, 2.5; 70, 8.1; and 149, 9.7.
[e] $1P < 0.05\%$.

Table 8D *Eight types of nonneoplastic liver lesions in control and NDMA-treated female rats that are strongly related to treatment: numbers of affected animals and tests for trend with respect to dose*

Treatment group	NDMA concentration (ppm)	No. of animals at risk[a]	No. of at-risk animals with: Hyperplastic nodules	Bile duct hyperplasia	Kupffer cell hyperplasia	Cytomegaly	Shrinkage of hepatocytes	Abnormality of glycogen-containing cells	Cysts[b]	Any of these lesions: Observed (O)	Expected (E)	Ratio (O/E)
1	0	219	14	6		7	36	11	7	64	81.7	0.8
2	0.003	55	8	1		2	13	1	4	20	23.0	0.9
3	0.066	53	3	0		2	10	2	2	19	20.8	0.9
4	0.132	53	3	1		0	12	4	2	18	20.0	0.9
5	0.264	50	4	1		2	8	1	7	16	19.2	0.8
6	0.528	48	4	2		0	9	3	3	19	17.2	1.1
7	1.056	41	6	1		6	9	4	5	22	15.4	1.4
8	1.584	15	0	0		0	1	1	4	5	4.3	1.2
9	2.112	16	2	1		2	1	5	3	9	4.9	1.8
10	2.640	9	1	0		0	1	7	1	8	2.8	2.9
11	3.168	5	0	0		0	2	3	2	5	1.8	2.8
12	4.224	4	0	0		1	0	3	2	3	1.2	2.5
13	5.280	2	1	0		2	0	1	1	2	0.6	3.1
14	6.336	1	0	0		0	0	0	0	0	0.2	0.0
15	8.448	3	1	0		1	0	2	3	3	0.4	7.2
16	16.896	1	1	0		0	0	0	0	1	0.2	5.1
Total (all doses)		575	48	13	0	25	102	48	46	214	214.0	1.0
Tests for positive trend:[d] 1P =			[e]	7.6%		[e]	1.5%	[e]	[e]	[e]		

[a] Numbers of rats whose livers came to full autopsy with no neoplasm(s), no diaphragmatic hernia, and no significant autolysis, cannibalism, or other loss of tissues to autopsy.
[b] Cysts, excluding any that were clearly due to lesions originating in the bile ducts. (Many of the remaining cysts were due to preneoplastic lesions of the blood vessels and thus contained some blood.)
[c] *O/E* values among the higher-dosed groups may suggest a misleadingly shallow dose-response relationship; see text.
[d] For statistical methods, see text or Ref. 3. "Death rate" methods were used for that minority of lesions which, by causing the liver to become palpable, led to sacrifice. "Prevelance methods" were used for the remainder, and the two analyses were combined in the usual way. The 8 pairs of T and z values that this generated were: 34, 7.2; 2, 1.5; –, –; 25, 6.4; 8, 2.2; 49, 7.3; 54, 10.1; and 89, 7.8.
[e] $1P \leq 0.05\%$.

agus, with further details provided only if a suggestive degree of statistical significance is found; Section 3, descriptions of the onset rates of both neoplastic and nonneoplastic lesions of the esophagus or liver at dose rates (*e.g.*, 1–2 ppm or less) so low that median times to death from the lesions of interest cannot be estimated reliably (since most animals will reach old age and die of unrelated causes, and only a small number will die of neoplasms of the liver or esophagus). Fortunately a corollary of the fact that in each of these low-dosed groups most animals die of unrelated conditions is that detailed statis-

Table 9 *Remaining nonneoplastic liver lesions*

Treatment group	Concentration (ppm)	No. of at-risk[a] animals with: Centrilobular necrosis NDEA M	NDEA F	NDMA M	NDMA F	Fatty foci/nodules NDEA M	NDEA F	NDMA M	NDMA F	Any other[b] nonneoplastic NDEA M	NDEA F	NDMA M	NDMA F
1	0	4	4	4	4	21	15	21	15	6	8	6	8
2	0.033	2	0	1	0	11	5	8	6	2	0	2	3
3	0.066	2	0	0	0	12	9	8	3	4	3	1	0
4	0.132	3	0	0	0	6	6	5	6	3	1	3	0
5	0.264	2	2	1	0	6	2	7	3	0	2	0	1
6	0.528	3	0	1	2	2	4	12	1	1	1	0	3
7	1.056	1	0	0	0	3	5	6	3	0	1	1	1
8	1.584	0	0	0	0	5	1	5	1	2	1	2	0
9	2.112	1	0	0	0	1	5	8	0	0	1	1	0
10	2.640	0	0	0	0	3	1	5	1	0	1	2	0
11	3.168	0	0	0	0	2	1	3	0	0	0	0	0
12	4.224	0	0	0	0	4	2	1	0	0	0	0	0
13	5.280	3	0	0	0	0	3	3	0	0	0	1	1
14	6.336	0	0	0	0	1	1	0	0	0	0	0	0
15	8.448	0	0	0	0	1	0	0	0	0	0	0	0
16	16.896	0	0	0	0	1	0	0	0	1	0	0	0
Total (all doses)		21	6	7	6	79	60	92	39	19	19	19	17
Test for positive trend:													
$1P$ =		NS[c]	NS	NS	NS	NS	0.1%	0.9%	NS	NS	NS	NS	NS
T =		−1	−1	−3	0	10	28	23	−2	6	3	4	1
z =		−0.1	−0.4	−1.1	−0.1	1.0	3.4	2.5	−0.5	1.0	1.1	0.9	0.2
Pooled (M and F) trend: $1P$ =		NS		NS		0.3%		2.5%		9.8%		NS	

[a] See Tables 8A to 8D for numbers at risk and for details of statistical method.
[b] This category excludes the 9 specific categories listed separately in Table 8 and includes various infective and other lesions. (Animals with this category of lesion may also, of course, have 1 of the 9 specific types as well.)
[c] NS, not significant.

tical correction for differences in longevity among different treatment groups becomes less important, and it becomes relatively straightforward to interpret the relationship with dose of the *total* numbers of animals observed in each group to have whatever type of lesion is of interest. (So, descriptions of dose-response relationships at low dose rates need emphasize only a classification of lesions by their biological nature and not by their "context of observation"; and, straightforward descriptions of dose-response relationships are possible not only for potentially life-threatening conditions such as neoplasms but also for various nonneoplastic liver lesions.); Section 4, finally, in a separate report (2) a formal "maximum likelihood" description of the various dose-response relationships (using "Weibull" distributions) will be presented. (Such a formal analysis can offer, at least to statisticians, a considerable simplification of the overall results, but it may appear to nonstatisticians to be inappropriately remote from the real data.)

1. Tumors of the Esophagus or Liver at High Dose Rates

1A. Esophagus. No control or NDMA-treated animal in the whole experiment was observed to develop any hyperplastic or neoplastic lesion of the esophagus; thus the following analysis of esophageal lesions is restricted to NDEA-treated rats. Figs. 3*A* and 3*B* give, for male and for female rats treated with NDEA, the times to death just from esophageal neoplasms. Casual comparison of the general appearances of Figs. 3*A* and 3*B* may misleadingly suggest that the effects of a given NDEA concentration on the esophagus are far greater for males than for females. However, this first impression conveys almost the exact reverse of the truth. In fact, among males and females treated with a given dose (in ppm v/v) of NDEA, the ratio of the esophageal cancer death rates *after a similar duration of treatment* is about 0.6[14] (male ÷ female), a ratio that is significantly less than unity. However, the corresponding ratio for NDEA-induced liver cancer mortality is only about 0.1[14] (male ÷ female). Since in the high dose range most NDEA-treated animals die either of esophageal or of liver cancer, any increase in the number of deaths from the one produces a corresponding decrease in the number of deaths from the other disease. The fact that the female excess is far more marked for liver cancer than for esophageal cancer thus means that, whereas similar numbers of NDEA-treated males die of each disease, the numbers of high NDEA-dosed females that die of liver cancer are much larger, so the number that die of esophageal cancer is correspondingly much smaller. This means that at each of the high NDEA concentrations too few females die of esophageal neoplasms to allow reliable direct estimation of median times to death from the disease. Thus a simple characterization of

[14] These sex ratios for the age-specific mortality rates were estimated from the results of tests for trend with respect to sex (female = 0, male = 1) stratified for treatment level (3). As noted previously ("Methods," Section 3B), if the test statistic for trend is written T, its standard error is written S and the mortality risk ratio (male ÷ female) is, at each age and in each treatment group, approximately equal to some constant R that is to be estimated, then $\log_e R \simeq T/S^2$ (and the standard error of this estimate of $\log_e R$ is approximately $1/S$). For example, for esophageal cancer mortality, $T = 14$ and $S = 5.3$, so $\log_e R \simeq -0.5 \pm 0.2$, whence $R \simeq 0.6$. Similar analyses for various types of liver neoplasm yielded:

Origin of fatal hepatoma	NDEA T	NDEA S	NDEA $\log_e R$	NDEA R	NDMA T	NDMA S	NDMA $\log_e R$	NDMA R
Liver cell	−253.5	9.9	−2.6 ± 0.1	0.1	−59.8	7.2	−1.1 ± 0.1	0.3
Bile duct	−2.7	1.1	−2.1 ± 0.9	0.1	−155.6	7.7	−2.6 ± 0.1	0.1
Mesenchyme	+0.4	1.8	+0.01 ± 0.5	1.1	−11.8	3.5	−1.0 ± 0.3	0.4
Kupffer cell	−1.3	0.8	−2.1 ± 1.3	0.1	−0.1	1.3	−0.1 ± 0.8	0.9
Any part	−259.1	10.3	−2.5 ± 0.1	0.1	−229.9	11.3	−1.8 ± 0.1	0.2

N.B.: Only for those sites (i.e., liver cells and bile ducts) where the standard error of $\log_e R$ is about 0.1 can the sex ratio be regarded as reasonably reliably known.

Table 10AB *Tests for dose-related trends for numbers of animals affected by various types of lesion: NDEA-treated rats and all controls*

Type of lesion	MN[a] or AN	A. Male			B. Female			AB. M and F
		No.[c] of rats affected	T = trend statistic	z = T/SE (T)	No.[c] of rats affected	T = trend statistic	z = T/SE (T)	(Pooled z and 1-tailed p[b])
Esophagus	MN	206	837.4	23.18	117	401.5	12.68	25.79[d]
	AN	410	1626.0	30.73	330	935.1	18.41	34.92[d]
Esophageal hyperplasia in absence of AN esophagus		53	213.7	13.17	73	348.6	12.96	17.90[d]
Liver	MN	273	1353.8	36.67	450	1951.5	52.28	62.96[d]
	AN	318	1362.8	37.36	528	2071.6	52.00	63.58[d]
Histological abnormality[e] of liver in absence of AN liver		377	211.1	7.71	250	190.3	11.62	12.58[d]
Nasopharynx	MN	3	6.4	3.17	0			3.17 ($1P \simeq 0.4\%$)
	AN	7	11.4	4.25	1	1.2	2.35	4.62[d]
Kidney	MN	4	1.3	1.74	1	0.8	2.37	2.58 ($1P \simeq 2.2\%$)
	AN	6	1.1	1.23	2	0.8	2.00	1.93 ($1P \simeq 4.7\%$)
Bladder or ureter	MN	2	2.0	2.78	1	0.2	1.70	3.01 ($1P \simeq 1.6\%$)
	AN	9	3.9	1.40	2	0.1	0.19	1.42 (NS)
Bronchus or lung	MN	1	−0.2	−0.38	0			−0.38 (NS)
	AN	3	−0.2	−0.32	0			−0.32 (NS)
Skin (including feet and ears)	MN	9	1.2	0.95	1	−0.0	−0.16	0.93 (NS)
	AN	11	1.3	0.96	1	−0.0	−0.16	0.94 (NS)
Subcutaneous tissue	MN	26	−0.5	−0.09	6	−1.8	−0.75	−0.39 (NS)
	AN	62	11.7	1.58	41	−3.3	−0.96	1.02 (NS)
Brain or other nervous tissues	MN	26	−3.9	−0.78	8	2.2	1.26	−0.32 (NS)
	AN	36	−7.3	−1.33	9	1.5	0.74	−0.99 (NS)
Lymphatic or hematopoietic tissues	MN	44	8.6	0.99	22	−2.0	−0.73	0.73 (NS)
	AN	58	8.9	1.01	24	−2.4	−0.87	0.71 (NS)
Mammary tissues	MN	5	1.8	0.93	28	0.9	0.44	0.95 (NS)
	AN	5	1.8	0.93	63	2.4	0.59	0.93 (NS)
Ovary	MN				3	−0.6	−0.95	−0.95 (NS)
	AN				8	4.0	2.28	2.28 ($1P \simeq 3.8\%$)
Uterus	MN				181	5.9	1.02	1.02 (NS)
	AN				216	6.6	0.81	0.81 (NS)
Testis	MN	2	−0.5	−0.87				−0.87 (NS)
	AN	46	−1.6	−0.47				−0.47 (NS)
Prostate, seminal vessels, or Cowper's complex	MN	3	1.5	2.21				2.21 ($1P \simeq 4.0\%$)
	AN	3	1.5	2.21				2.21 ($1P \simeq 4.0\%$)
Harderian gland	MN	2	2.4	1.18	0			1.18 (NS)
	AN	3	3.6	1.66	0			1.66 (NS)
Pituitary	MN	5	0.3	0.13	2	0.2	0.10	0.16 (NS)
	AN	132	5.7	0.87	208	−9.2	−1.30	−0.36 (NS)
Adrenal	MN	8	−0.2	−0.26	4	1.1	1.04	0.65 (NS)
	AN	14	−2.2	−1.18	10	1.5	0.86	−0.26 (NS)
Thymus	MN	8	−1.8	−0.49	24	−0.3	−0.15	−0.50 (NS)
	AN	14	−2.9	−0.78	44	−0.8	−0.32	−0.83 (NS)
Thyroid	MN	14	0.3	0.17	7	−0.5	−0.83	−0.06 (NS)
	AN	51	0.4	0.09	25	−1.8	−1.31	−0.36 (NS)
Lower jaw, tongue, and salivary gland	MN	19	6.25	2.46	6	0.04	0.03	2.14 ($1P \simeq 2.7\%$)
	AN	24	7.0	2.53	12	−0.5	−0.29	2.05 ($1P \simeq 3.0\%$)
Pancreas	MN	4	−0.4	−0.67	0			−0.67 (NS)
	AN	11	−2.8	−1.03	2	0.1	0.82	−0.97 (NS)
Stomach	MN	3	4.5	2.21	2	1.7	3.03	2.92 ($1P \simeq 1.4\%$)
	AN	4	9.0	2.97	4	0.6	0.46	2.92 ($1P \simeq 0.8\%$)
Small intestine	MN	5	−0.6	−0.74	2	−0.2	−0.39	−0.83 (NS)
	AN	13	0.2	0.17	4	−0.4	−0.76	−0.16 (NS)
Large intestine (including cecum, rectum)	MN	0			1	−0.1	−0.59	−0.59 (NS)
	AN	2	−0.3	−0.76	1	−0.1	−0.59	−0.94 (NS)

Table 10AB *Continued*

Type of lesion	MN[a] or AN	A. Male No.[c] of rats affected	A. Male T = trend statistic	A. Male z = T/SE (T)	B. Female No.[c] of rats affected	B. Female T = trend statistic	B. Female z = T/SE (T)	AB. M and F (Pooled z and 1-tailed p[b])
Remaining sites (chiefly, various connective tissues)	MN	13	−3.2	−1.17	7	0.8	0.29	−0.64 (NS)
	AN	32	−3.6	−1.18	11	−0.0	−0.01	−0.88 (NS)
Any neoplasm, except of liver, esophagus, nasopharynx	MN	183	13.6	1.00	263	3.8	0.47	1.10 (NS)
	AN	403	8.2	0.50	490	−11.6	−0.98	−0.17 (NS)
Sacrificed after palpation error		12	35.8	5.22	6	29.1	4.18	6.64[d]
Deaths not attributed to neoplasms[f] (excluding scheduled sacrifice or sacrifice after positive palpation)								
(a) Tissues lost to autopsy[f]		9	−13.4	−1.43	8	23.5	3.33[g]	0.86 (NS)
(b) Tissues partially lost		5	0.5	0.23	7	0.4	0.27	0.34 (NS)
(c) Full autopsy		201	−15.3	−0.94	48	−8.2	−1.72	−1.39 (NS)

[a] MN, malignant neoplasm of; AN, any neoplasm of; NS, not statistically significant ($2P > 0.05$).
[b] See "Appendix 1" for method of estimation of 1-tailed P values.
[c] Excludes all rats with congenital diaphragmatic hernia and excludes incidental tumors in rats with relevant (see text) autolysis or cannibalism.
[d] Estimated (see Footnote *b*) 1-tailed $P < 0.05\%$.
[e] See Table 8 for details.
[f] Includes all deaths where a diaphragmatic hernia was discovered.
[g] $1P = 0.7\%$, $2P = 1.4\%$.

the dose-response relationship for esophageal lesions is possible only from the data for males. Fig. 4 therefore gives, for *male* rats only, the relationship of NDEA dose rate (in ppm v/v) to the median time to death just from esophageal neoplasms.

In Fig. 4, "logarithmic" scales are used along each axis of the graph. Because of this, the points corresponding to doses above 2 ppm all lie approximately on a straight line. Moreover, the uncertainty in the data points at 1–2 ppm is such that they too do not lie significantly above this line. The line suggested by these data is approximately that for which a 5-fold increase in NDEA concentration is needed to produce a 2-fold decrease in median time to death just from esophageal neoplasm(s).

The carcinogenic potency implied by the dose-response relationship in Fig. 4 is quite remarkable. After exposure for a standard life span of 2 years to only 2 ppm (v/v; see "Methods," Section 8) of NDEA, the cumulative mortality from esophageal cancer is such that, even in the absence of all other causes of death, about one-half of the males would have died of it, and of course, after 2 years of treatment many more would already have developed preneoplastic or neoplastic lesions of the esophagus that have not (yet) caused death.

This latter point is illustrated by the detailed observed and expected numbers in Tables 5A (males) and 5B (females) of animals with various types of esophageal lesions, observed in various contexts. Even among males the number of nonfatal esophageal neoplasms is nearly as great as the number of fatal ones, whereas among females (where, at high dose levels, death from hepatomas usually occurred before the esophageal neoplasms had had time to develop to a life-threatening type and/or size), the number of nonfatal esophageal neoplasms (271) considerably exceeds the number of fatal ones (58).

In Table 5 there is no clear trend with dose in the proportions of the esophageal lesions that are hyperplastic, benign, or malignant, which indicates that the general forms of the three dose-response relationships for these three types of lesion are rather similar.

In Table 5A (males), the steady progression in the O/E ratios for fatal esophageal neoplasms reflects the regularity of the dose-response relationship in Fig. 4 (and, as has already been noted in "Statistical Methods," the lack of any marked progression at high dose levels in the O/E ratios for the incidental lesions in Tables 5A and 5B is not particularly informative). In Table 5B the progression in the O/E ratios for females is somewhat less regular, but this is presumably merely because the numbers of females dying of the disease are so small that chance has a relatively large effect.

1B. Liver. The description of the response of the liver to the test nitrosamines is more complex than was description of the response of the esophagus for several reasons: (*a*) both agents (NDEA and NDMA) cause liver neoplasms; (*b*) their quantitative effects on males and on females are more markedly different; (*c*) four main histological categories of liver neoplasm were recorded—liver cell, bile duct, "mesenchymal"[15] (chiefly blood vessel, of course) and, least commonly, Kupffer cell—and the proportions in which these four categories of neoplasm occur depend strongly on dose, on sex, and on nitrosamine (NDEA/NDMA); (*d*) several different categories of nonneoplastic liver lesion were described, most (but not all) of which were strongly dose related; (*e*) whereas the background rates of spontaneous esophageal neoplasms were negligible, this is not true of the background rates of most types of neoplastic and of nonneoplastic liver lesions: among the animals that were not[16] scheduled to be sacrificed at 12 or at 18 months, for example, liver neoplasms were detected chiefly in old age, of course, among 8% of the controls (8 malignant plus 21 benign out of 384 animals, one-half of each sex) and among 10% of the animals in the two lowest dose groups (8 malignant plus 30 benign out of 384).

Restricting attention initially to *neoplastic* liver lesions, Fig. 6 describes the cumulative mortality from the totality of all neoplastic lesions (benign or malignant, liver cell, bile duct, mesenchyme, or Kupffer). The higher treatment levels caused a sufficient number of fatal liver neoplasms to allow the median time to death just from these diseases to be estimated reasonably reliably from Fig. 6, and the relationship between these medians and the dose levels to which they correspond is presented in Fig. 7. At any particular dose level (expressed in ppm v/v), the

[15] "Mesenchymal" tumors comprised all the hemangiomas, *i.e.*, the sarcomas, pericytomas, and endotheliomas of the blood vessels, probably although not definitely arising chiefly from the sinusoidal blood vessels.
[16] Because spontaneous liver neoplasms tend to arise only in extreme old age, none was found at the scheduled sacrifices at 12 or 18 months in the control group or the two lowest-dosed groups.

Table 10CD *Tests for dose-related trends for numbers of animals affected by various types of lesion: NDMA-treated rats and all controls*

Type of lesion	MN[a] or AN	C. Male No.[c] of rats affected	C. Male T = trend statistic	C. Male z = T/SE (T)	D. Female No.[c] of rats affected	D. Female T = trend statistic	D. Female z = T/SE (T)	CD. M and F (pooled z and 1-tailed p[b])
Esophagus	MN	0			0			
	AN	0			0			
Esophageal hyperplasia in absence of AN esophagus		0			0			
Liver	MN	292	1666.6	46.11	188	1297.4	39.33	60.57[d]
	AN	414	1999.5	49.72	547	2259.9	54.00	73.39[d]
Histological abnormality[e] of liver in absence of AN liver		303	148.5	9.72	214	94.0	8.18	12.68[d]
Nasopharynx	MN	0			1	−0.2	−0.25	−0.25 (NS)
	AN	0			1	−0.2	−0.25	−0.25 (NS)
Kidney	MN	8	2.5	0.53	3	−2.0	−0.43	0.07 (NS)
	AN	10	1.8	0.39	5	−1.3	−0.27	0.08 (NS)
Bladder or ureter	MN	1	1.3	0.56	0			0.56 (NS)
	AN	9	5.0	0.65	1	−0.3	−0.61	0.61 (NS)
Bronchus or lung	MN	5	2.4	0.97	0			0.97 (NS)
	AN	7	10.3	2.85	1	0.9	2.92	3.09 (1$P \simeq$ 0.4%)
Skin (including feet and ears)	MN	8	4.5	1.79	1	−0.2	−0.49	1.69 (NS)
	AN	11	8.6	2.70	2	−0.2	−0.55	2.60 (1$P \simeq$ 1.1%)
Subcutaneous tissue	MN	30	4.0	0.62	13	−2.2	−0.77	0.26 (NS)
	AN	73	10.3	1.06	51	−2.0	−0.38	0.74 (NS)
Brain or other nervous tissues	MN	27	1.7	0.2	11	−0.1	−0.05	0.20 (NS)
	AN	41	−6.8	−0.82	13	1.4	0.49	−0.60 (NS)
Lymphatic or hematopoietic tissues	MN	35	−1.6	−0.20	25	19.5	3.43	1.79 (NS)
	AN	48	−0.6	−0.07	30	21.8	3.74	2.00 (1$P \simeq$ 3.2%)
Mammary tissues	MN	5	−1.3	−0.51	29	2.8	0.64	0.30 (NS)
	AN	6	−0.1	−0.03	74	4.1	0.57	0.52 (NS)
Ovary	MN				7	−1.9	−1.26	−1.26 (NS)
	AN				12	0.6	0.29	0.29 (NS)
Uterus	MN				188	−1.5	−0.17	−0.17 (NS)
	AN				234	5.8	0.48	0.48 (NS)
Testis	MN	2	−1.2	−0.92				−0.92 (NS)
	AN	39	5.6	0.96				0.96 (NS)
Prostate, seminal vessels, or Cowper's complex	MN	5	8.8	3.01				3.01 (1$P \simeq$ 0.7%)
	AN	8	10.1	3.21				3.21 (1$P \simeq$ 0.4%)
Harderian gland	MN	1	−0.7	−0.59	0			−0.59 (NS)
	AN	1	−0.7	−0.59	0			−0.59 (NS)
Pituitary	MN	3	−0.3	−0.14	0			−0.14 (NS)
	AN	185	12.7	0.93	223	2.5	0.23	0.86 (NS)
Adrenal	MN	12	1.8	0.69	4	2.8	1.56	1.46 (NS)
	AN	21	3.3	0.79	9	3.7	1.34	1.39 (NS)
Thymus	MN	8	−3.1	−0.81	38	3.1	0.62	0.00 (NS)
	AN	14	−1.2	−0.25	62	0.1	0.02	−0.14 (NS)
Thyroid	MN	18	0.1	0.03	5	−0.3	−0.26	−0.03 (NS)
	AN	73	−5.7	−0.68	20	−3.8	−1.39	−1.08 (NS)
Lower jaw, tongue, and salivary gland	MN	16	−7.4	−1.45	10	−2.5	−1.02	−1.75 (NS)
	AN	25	−3.2	−0.53	22	−0.4	−0.09	−0.49 (NS)
Pancreas	MN	7	−2.3	−1.36	0			−1.36 (NS)
	AN	19	1.9	0.55	1	−0.1	−0.41	0.51 (NS)
Stomach	MN	4	1.0	0.35	4	−2.8	−1.07	−0.48 (NS)
	AN	6	7.6	2.29	6	−4.6	−1.52	0.65 (NS)
Small intestine	MN	7	2.1	0.80	0			0.80 (NS)
	AN	16	1.1	0.24	4	−0.8	−0.87	0.06 (NS)
Large intestine (including cecum, rectum)	MN	0			2	0.5	0.87	0.87 (NS)
	AN	2	−0.8	−0.91	2	0.5	0.87	−0.22 (NS)

Table 10CD *Continued*

Type of lesion	MN[a] or AN	C. Male No.[c] of rats affected	C. Male T = trend statistic	C. Male z = T/SE (T)	D. Female No.[c] of rats affected	D. Female T = trend statistic	D. Female z = T/SE (T)	CD. M and F (pooled z and 1-tailed p[b])
Remaining sites (chiefly, various connective tissues)	MN	18	5.8	1.48	13	2.4	0.70	1.58 (NS)
	AN	42	5.6	1.02	18	3.4	0.83	1.31 (NS)
Any neoplasm, except of liver, esophagus, nasopharynx	MN	197	11.6	0.67	297	15.0	1.06	1.19 (NS)
	AN	495	23.4	0.94	551	21.7	1.11	1.43 (NS)
Sacrificed after palpation error		6	5.9	1.15	5	15.5	3.54	3.16 ($1P \simeq 0.4\%$)
Deaths not attributed to neoplasms[f] (excluding scheduled sacrifice or sacrifice after positive palpation)								
(a) Tissues lost to autopsy[f]		13	−7.5	−0.80	10	13.6	2.70	0.66 (NS)
(b) Tissues partially lost		3	3.3	0.85	6	1.7	0.52	0.98 (NS)
(c) Full autopsy		224	16.5	0.95	53	−10.0	−1.07	0.33 (NS)

[a] MN, malignant neoplasm of; AN, any neoplasm of; NS, not statistically significant ($1P > 0.05$).
[b] See "Appendix 1" for methods of estimation of 1-tailed P values.
[c] Excludes all rats with congenital diaphragmatic hernia and excludes incidental tumors in rats with relevant (see text) autolysis or cannibalism.
[d] Estimated (see Footnote *b*) 1-tailed $P < 0.05\%$.
[e] See Table 8 for details.
[f] Includes all deaths where a diaphragmatic hernia was discovered.

Table 11 *Sites (other than liver or esophagus) at which a suggestively[a] positive trend was observed*

No. of table with details	Site of neoplasm	Trend with NDEA[b] No.[c]	Trend with NDEA[b] z	Trend with NDEA[b] $1P$ (%)	Trend with NDMA[b] No.[c]	Trend with NDMA[b] z	Trend with NDMA[b] $1P$ (%)
12	Nasopharynx	8	4.62	<0.1	1	−0.25	NS[d]
13	Lower jaw, etc.	36	2.05	3.0	47	−0.49	NS
14	Stomach	8	2.92	0.8	12	0.65	NS
15	Lung, etc.	3	−0.32	NS	8	3.09	0.4
16	Kidney	8	1.93	4.7	15	0.08	NS
17	Skin, etc.	12	0.94	NS	13	2.60	1.1
18	Bladder or ureter	11	1.42	NS	10	0.61	NS
19A	Ovaries	8	2.28	3.8	12	0.29	NS
19B	Seminal vessels, etc.	3	2.21	4.0	8	3.21	0.4
20	Lymphatic/hematopoietic	82	0.71	NS	78	2.00	3.2

[a] *i.e.*, $z \geq 2.0$ in the pool of the analyses of males and of females for any of (1) NDEA, malignant neoplasms, (2) NDEA, any neoplasms, (3) NDMA, malignant neoplasms, or (4) NDMA, any neoplasms.
[b] The tabulated analyses are for any neoplasm (*i.e.* benign or malignant) and derive from the pool of the analyses of males and of females. For sex-specific analyses or for analyses limited to malignant neoplasms only, see Table 10.
[c] Number of affected animals in control group plus groups treated with specified agent.
[d] NS, not significant ($1P > 0.05$).

female rates were higher than the male rates (for details, see Footnote 14) and even though conversion of the units of dosage to mg/kg/day (see "Methods," Section 8) would bring these male and female lines somewhat closer together, it would not cause them to overlap.

Comparison of Fig. 7*AB* with Fig. 7*CD* suggests that in animals of the same sex the effects of NDEA and NDMA on *total* liver tumorigenesis are quantitatively rather similar (and conversion from ppm v/v into molar units would make them almost identical). However, this similarity is perhaps somewhat fortuitous, for on separate examination of the different histological types of liver tumor, gross differences are seen. The fatal NDEA-induced hepatomas, for example, were nearly all malignant liver cell hepatomas, while only about one-half of the fatal NDMA-induced hepatomas were, the remainder being benign neoplasms of the intrahepatic bile ducts.

Table 6 provides some histological details of the *fatal* liver neoplasms separately for: A, control and NDEA-treated males; B, control and NDEA-treated females; C, control and NDMA-treated males; D, control and NDMA-treated females. Table 7 does likewise for the *incidental* liver neoplasms.

Nearly all of the liver cell, mesenchymal, and Kupffer cell neoplasms that were detected were malignant, while nearly all of the bile duct neoplasms were benign. Thus, if the four different cell types of origin are considered separately, it is not practicable to subdivide these four analyses further according to whether the lesions concerned were benign or malignant, for in each case one or the other of these subdivisions would contain nearly all of the data and the other would contain too little data to be reliable.

Likewise, because the proportion of liver neoplasms that were found in an incidental context was small, the general features of the dose-response relationship will be rather similar whether Table 6 is viewed alone or in conjunction with Table 7. For reasons that were discussed in Section 4, in the high dose range, inference will be based chiefly just on the fatal tumors (Table 6) while, in the low dose range, inference will be based chiefly on all tumors (Tables 6 and 7).

For both the mesenchymal and the Kupffer cell tumors in Table 6 the relationship with dose is reasonably regular, and for each it is fairly similar for all four combinations of NDEA, NDMA, male, and female, once differences in longevity and in intake in μmol/day (see "Methods," Section 8) have been allowed for.

For the liver cell tumors in Table 6, the effects of treatment are illustrated in Fig. 8. Both for NDMA and, especially, for NDEA these effects are greater for females than for males (for details, see Footnote 14). However, substantial numbers of liver

Table 12 *Observed and expected numbers of animals with a tumor of the nasopharynx*

None were fatal. Such tumors were sought after decalcification of all available skulls.

Treatment group	Concentration (ppm)	Control and NDEA		Control and NDMA	
		Expected	Observed[a]	Expected	Observed[a]
1	0	2.35	0	0.34	0
2	0.033	0.60	0	0.09	0
3	0.066	0.64	0	0.08	0
4	0.132	0.61	0	0.09	0
5	0.264	0.55	0	0.10	1 (F)
6	0.528	0.60	0	0.08	0
7	1.056	0.59	2 (2m)	0.09	0
8	1.584	0.54	2 (m, f)	0.06	0
9	2.112	0.38	1 (M)	0.05	0
10	2.640	0.35	1 (M)	0.01	0
11	3.168	0.36	0	0.01	0
12	4.224	0.28	1 (M)	—	—
13	5.280	0.14	1 (m)	—	—
14	6.336	0.01	0	—	—
15	8.448	—[b]	—	—	—
16	16.896	—	—	—	—
Total (all doses)		8.00	8	1.00	1
Tests for trend		$z = 4.62$ $1P < 0.1\%$		$z = -0.26$ Not significant	

[a] Key to specification of sex and of histology: m, male, benign; f, female, benign; M, male, other; F, female, other.

[b] —, a group in which all the animals were dead before the first such neoplasm was discovered in any other group (and in which, therefore, the expected and observed numbers are both necessarily zero).

cell tumors are caused in each sex by each nitrosamine.

For intrahepatic bile duct tumors the effects are also greater for females than for males, but in contrast with the liver cell tumors (which were strongly affected by both NDMA and NDEA) bile duct tumors are induced far more readily by NDMA than by NDEA. Indeed, not a single one of the 960 animals that received a high dose rate of NDEA died of a bile duct neoplasm, whereas nearly 300 did so among the 960 animals that received a high dose rate of NDMA. The relationship of NDMA dose rate to cumulative mortality from bile duct neoplasms is illustrated in Fig. 9, and at each (high) dose level the excess among females is substantial.

Fig. 10 plots medians from Fig. 8 for hepatocellular neoplasms. Again, it is clear that at each dose level of NDEA the females responded more rapidly than the males, although if dose were expressed in mg/kg/day (see "Methods," Section 8) instead of in ppm this difference would be narrowed. The range of NDMA doses for which the median times to death from such neoplasms can be read directly from Fig. 8 is limited to the extremely high dose levels, because at intermediate NDMA dose levels so many animals die prematurely of bile duct neoplasms that few have time to die of hepatocellular neoplasms. Despite the limited range of NDMA doses in Fig. 10, however, the slope of the NDMA dose-response relationship in Fig. 10*CD* appears to be far steeper than that of the NDEA dose-response relationship in Fig. 10*AB*. The slopes are approximately −1 for NDMA-induced hepatocellular tumors, and −1 in about 2.3 for NDEA-induced such tumors. This difference in slopes means that to halve the median time to death just from hepatocellular tumors, one would need about a 2-fold increase in NDMA dose but about a 5-fold increase in NDEA dose, perhaps suggesting some important difference in the mechanisms of hepatocellular carcinogenesis by these two agents.

Fig. 11*CD* plots medians from Fig. 9 for bile duct neoplasms in NDMA-treated animals. (So few bile duct neoplasms were induced by NDEA that no such plot of the effects of NDEA is possible.) The difference between NDMA-treated males and females is even more marked for bile duct neoplasms than for hepatocellular neoplasms, and another important difference is in the slopes for the two diseases: that for NDMA-induced bile duct neoplasms being −1 in about 2.3. The difference between the slopes of the dose-response relationships for bile duct and for hepatocellular neoplasms is emphasized in Fig. 12*D*; if Fig. 12*D* is too far removed from the data for the taste of some readers, this marked difference between the two dose-response relationships is also apparent if Fig. 8*D* is simply compared visually with Fig. 9*D*. Clearly, within the top eight or so treatment levels changes in the NDMA concentration have a far larger effect on the onset rates of hepatocellular carcinomas than on the onset rates of bile duct neoplasms. Indeed, in the high dose range the effects of changes in the dose rate on mortality from bile duct neoplasms is really quite slight (Fig. 9*D*). It is all the more surprising, therefore, to note how sharply the effects on bile duct neoplasm induction in females of 1.056 and 1.584 ppm of NDMA appear to differ, the former causing just 1 death and the latter 25 (Table 6D). This contrast is, however, probably greatly inflated by the play of chance, for no such sharp cutoff is seen among males or among the numbers of incidental bile duct tumors (Table 7), so it is unlikely to indicate any truly important threshold effect (see subsequent sections on dose-response relationships in the low dose range).

Because of the flatness of the dose response for bile duct neoplasms compared with the steepness of that for hepatocellular neoplasms (Fig. 12*D*), the predominant histology of the fatal liver tumors induced by NDMA in females changes from hepatocellular neoplasms at the highest doses to bile duct neoplasms at intermediate doses, and the shape of the dose response for total liver tumor mortality (*i.e.*, any cell type) reflects a complex combination of the shapes of the separate dose-response relationships.

It is difficult to interpret these separate relationships biologically unless some measure of the effective dose of the test agent at its exact target (bile duct, parenchymal cell, etc.) is available, and even then the effective carcinogenic insult may be modified by dose-rate-dependent effects on metabolic activation enzymes or on other enzymes.

As a further source of complexity, Table 8 lists the numbers of animals in which various treatment-related nonneoplastic lesions were found in the routine liver sections. Throughout the high dose range the prevalence of certain lesions is elevated, thereby perhaps significantly changing the biological environment in which the cells of the liver exist. This widespread disruption of the normal architecture of the liver may well itself affect the likelihood that a cell that has been exposed to nitrosamines will progress to full neoplastic transformation, thereby further complicating the dose-response relationship for tumors.

Finally, Table 9 describes the numbers of animals with certain types of liver lesion that were not as strongly related to treatment.

2. *Tumors of Sites Other Than the Liver or Esophagus*

Fig. 2 described mortality from the aggregate of all diseases except of the esophagus or liver (and, of course, except for scheduled sacrifice, or sacrifice on suspicion of a palpable liver lump). No clear treatment-related trends were evident. Likewise, no significant treatment-related trends are evident in mortality from the aggregate of all remaining tumors other than

Table 13 *Observed and expected numbers of animals with tumors of the oral cavity (chiefly squamous carcinomas of the lower jaw)*

Treatment group	Concentration (ppm)	Control and NDEA		Control and NDMA	
		Expected	Observed[a]	Expected	Observed[a]
1	0	13.96	14 (4M, M,[b] m,[c] 3F)	15.09	14 (4M, M,[b] m,[c] 3F, 5f)
2	0.033	3.37	2 (M, m)	4.46	4 (2M, M,[c] F)
3	0.066	3.69	0	3.80	4 (M, 2m, F)
4	0.132	3.45	2 (2M)	3.79	7 (3M, 2F, 2f)
5	0.264	3.47	4 (3M, f)	3.82	5 (m, 2F, 2f)
6	0.528	3.32	6 (2M, 2m, 2F)	3.61	2 (m, f)
7	1.056	2.46	4 (2M, m,[b] F)	3.53	3 (M, F, f)
8	1.584	1.32	2 (M,[b] M[c])	2.49	3 (3M)
9	2.112	0.46	1 (M)	2.55	2 (2m)
10	2.640	0.25	0	1.30	2 (2m)
11	3.168	0.12	1 (M)	1.17	0
12	4.224	0.09	0	0.79	0
13	5.280	0.03	0	0.27	0
14	6.336	—	—	0.22	1 (f)
15	8.448	—	—	0.09	0
16	16.896	—	—	—	—
Total (all doses)		36.00	36	47.00	47
Tests for trend		$z = 2.05$ $1P \simeq 3.0\%$		$z = -0.49$ Not significant	

[a] See footnotes to Table 12.
[b] Tongue.
[c] Salivary gland.

of the liver or esophagus, or in the prevalence of these remaining tumors among animals that died of nonneoplastic causes (or of liver or esophageal neoplasms).

Depending on whether the analysis of these remaining (non-liver, nonesophageal) tumors is of males only, of females only, or of (by addition) both, on whether it is with respect to NDEA dose or to NDMA dose, and on whether it relates to malignant or to all such neoplasms, a total of 12 ($3 \times 2 \times 2$) different statistical tests are possible for a trend with respect to dose in the difference between the observed and expected numbers of animals with a neoplasm at some site(s) other than the liver or esophagus. Although nearly 2000 animals developed some such neoplasm(s) (about one-half of whom had at least one malignant such neoplasm), all 12 of these tests for trend were wholly nonsignificant, with z values between -1.0 and $+1.2$ (each $1P > 0.1$). This indicates that, as expected, the absolute increase in tumor onset rates is large only for neoplasms of the liver and (with NDEA) the esophagus, but it does not guarantee that no

Table 14 *Observed and expected numbers of animals with tumors of the stomach (chiefly squamous carcinomas)*

Treatment group	Concentration (ppm)	Control and NDEA		Control and NDMA	
		Expected	Observed[a]	Expected	Observed[a]
1	0	2.71	4 (M, F, 2f)	3.60	4 (M, F, 2f)
2	0.033	0.67	0	1.13	1 (F)
3	0.066	0.71	1 (M)	0.98	2 (M, F)
4	0.132	0.73	0	0.87	1 (M)
5	0.264	0.71	0	0.91	0
6	0.528	0.69	0	0.90	1 (F)
7	1.056	0.55	0	0.83	0
8	1.584	0.34	0	0.58	0
9	2.112	0.21	1 (F)	0.67	0
10	2.640	0.18	0	0.40	0
11	3.168	0.16	0	0.38	1 (m)
12	4.224	0.12	0	0.30	1 (M)
13	5.280	0.10	0	0.19	1 (m)
14	6.336	0.09	2 (M, m)	0.18	0
15	8.448	0.04	0	0.08	0
16	16.896	—	—	—	—
Total (all doses)		8.00	8	12.00	12
Tests for trend		$z = 2.92$ $1P = 0.8\%$		$z = 0.65$ Not significant	

[a] See footnotes to Table 12.

Table 15 *Observed and expected numbers of animals with tumors of the lung, etc.*

Treatment group	Concentration (ppm)	Control and NDEA		Control and NDMA	
		Expected	Observed[a]	Expected	Observed[a]
1	0	1.24	0	2.37	0
2	0.033	0.18	0	0.74	0
3	0.066	0.45	1 (m)	0.67	0
4	0.132	0.31	1 (M[b])	0.60	0
5	0.264	0.29	1 (m)	0.64	1 (M)
6	0.528	0.22	0	0.61	0
7	1.056	0.18	0	0.56	3 (2M, f)
8	1.584	0.11	0	0.43	1 (M)
9	2.112	0.02	0	0.45	2 (M,[b] m)
10	2.640	0.01	0	0.27	0
11	3.168	—	—	0.26	0
12	4.224	—	—	0.19	0
13	5.280	—	—	0.09	0
14	6.336	—	—	0.08	0
15	8.448	—	—	0.04	1 (m)
16	16.896	—	—	—	—
Total (all doses)		3.00	3	8.00	8
Tests for trend		$z = -0.32$ Not significant		$z = 3.09$ $1P \simeq 0.4\%$	

[a] See footnotes to Table 12.
[b] Bronchus.

clear increases will be found in some remaining type(s) of tumor, especially those which are extremely rare among untreated animals. It does, however, underline the need for caution in accepting as real without independent evidence[17] any effects that are suggested by the data, because with two agents, two sexes, and a couple of dozen anatomic sites to study, a few moderately significant false positives can be expected by chance alone.

As a first step toward deciding which other sites are most worthy of closer scrutiny, Table 10 lists the results of the standard (trend) tests of statistical significance for each of many sites. The most important column is perhaps the final one, in which a pooled z value for male and female animals is presented, together with its corresponding P value. The sites for which this pooled z value achieves a value of at least 2.0 (either for

[17] For example, of tissue-specific DNA damage, or of consistency between the two sexes in the tissues apparently affected, or of similarity with the tissues affected by some other nitrosamine(s).

Table 16 *Observed and expected numbers of animals with tumors of the kidney (chiefly carcinomas)*

Treatment group	Concentration (ppm)	Control and NDEA		Control and NDMA	
		Expected	Observed[a]	Expected	Observed[a]
1	0	3.29	3 (M, m,[b] f[b])	4.46	3 (M, m,[b] f[b])
2	0.033	0.68	0	1.36	2 (M,[c] m)
3	0.066	0.95	3 (M, M,[c] m)	1.24	1 (F)
4	0.132	0.94	0	0.97	0
5	0.264	0.78	0	1.10	2 (2M)
6	0.528	0.80	0	1.08	2 (2M)
7	1.056	0.41	1 (f)	1.07	2 (F, f[b])
8	1.584	0.12	0	0.69	1 (M)
9	2.112	0.02	1 (M[c])	0.72	1 (F[c])
10	2.640	0.01	0	0.55	0
11	3.168	—	—	0.50	0
12	4.224	—	—	0.45	0
13	5.280	—	—	0.30	0
14	6.336	—	—	0.28	0
15	8.448	—	—	0.18	1 (M[c])
16	16.896	—	—	0.04	0
Total (all doses)		8.00	8	15.00	15
Tests for trend		$z = 1.93$ $1P \simeq 4.7\%$		$z = 0.08$ Not significant	

[a] See footnotes to Table 12.
[b] Undetermined cell type of origin.
[c] Mesenchymal.

Table 17 *Observed and expected numbers of animals with tumors of the skin*

Treatment group	Concentration (ppm)	Control and NDEA		Control and NDMA	
		Expected	Observed[a]	Expected	Observed[a]
1	0	4.79	2 (2M)	4.17	2 (2M)
2	0.033	0.95	2 (M,[b] m)	1.39	1 (F)
3	0.066	1.41	2 (M, F)	1.07	3 (2M,[b,c] f)
4	0.132	1.35	1 (M[b])	1.08	0
5	0.264	1.15	0	1.08	1 (m)
6	0.528	1.28	4 (3M, m)	1.12	0
7	1.056	0.70	0	0.89	0
8	1.584	0.22	1 (M)	0.60	1 (M)
9	2.112	0.07	0	0.74	2 (2M)
10	2.640	0.05	0	0.38	0
11	3.168	0.02	0	0.26	3 (M, 2m)
12	4.224	—	—	0.20	0
13	5.280	—	—	0.02	0
14	6.336	—	—	0.01	0
15	8.448	—	—	—	—
16	16.896	—	—	—	—
Total (all doses)		12.00	12	13.00	13
Tests for trend		$z = 0.94$ Not significant		$z = 2.60$ $1P \simeq 1.1\%$	

[a] See footnotes to Table 12.
[b] Feet.
[c] Ear.

"malignant neoplasms" or for "any neoplasms") are listed in Table 11, which is perhaps the key table to examine. For these sites, details of the observed and expected numbers of tumor-bearing animals are provided in Tables 12–20.

The only site for which the trend is so overwhelmingly strong that it cannot reasonably be suggested that the apparent association might be a "false positive" is the nasopharynx, which appears (like the esophagus) to be clearly affected by NDEA but not by NDMA.

For some of the remaining sites the "statistically significant trend" with respect to one type of nitrosamine that is noted in Table 11 appears (on comparison with the onset rates in the aggregate of all animals treated with the other type of nitrosamine, for which no trend was evident) to be due chiefly to a shortage of affected animals at the lower dose levels rather than to an excess at the higher dose levels.

Thus, for example, on examination of Table 16 we find that the "statistically significant" ($P < 0.05$) association of NDEA with kidney tumor onset rates is "significant" just because at treatment levels 2–6 (which, to judge by the dose-response relationships for liver and esophageal cancer, are unlikely to have any substantial effect) only 3 NDEA-treated animals developed spontaneous kidney neoplasms while 7 NDMA-treated animals did so. This is not suggestive of any real carcinogenic effect of NDEA.

Similar arguments may be deployed against the association of NDEA with tumors of the lower jaw (Table 13) or stomach (Table 14), although these two associations might, in view of the marked effect of NDEA on the esophagus, have been thought to be among the more plausible data-derived hypotheses in Table 11, and against the association of NDMA with cancers of the skin (Table 17), of the ovary (Table 19), and of the reticuloendothelial system (Table 20). Moreover, in other experiments even repeated applications of these nitrosamines to the skin have failed to elicit skin tumors (1), while in the present experiment an association of treatment with reticuloendothelial neoplasms is seen only for NDMA-treated females (Table 20), and not for NDMA-treated males or for NDEA in either sex, which makes a cause-and-effect explanation for the observed association less plausible than if a more generalized effect were seen.

Indeed, the only two associations in Table 11 that have the characteristics one might expect of a real cause-and-effect relationship are those of NDMA with the seminal vessels (Table 19) and the lungs (Table 15). Although the former has no obvious biological rationale and thus may be a somewhat extreme artifact of chance, the latter is quite plausible, for NDMA has been shown to be a lung carcinogen in other species.

Thus, of the apparent effects listed in Table 11 only that of NDEA on the nasopharynx and, perhaps, NDMA on the lungs can be regarded as established by this experiment. However, although the remaining effects are not of themselves convincing, that does not prove that they are not real, and if these associations were independently supported by other evidence then judgment as to the most plausible interpretation of these findings might change. This would be particularly true for tumors

Table 18 *Observed and expected numbers of animals with tumors of the bladder, etc.*

Treatment group	Concentration (ppm)	Control and NDEA		Control and NDMA	
		Expected	Observed[a]	Expected	Observed[a]
1	0	4.44	5 (4m, f)	3.03	5 (4m, f)
2	0.033	0.74	0	0.87	0
3	0.066	1.26	1 (m)	0.96	0
4	0.132	1.16	0	0.58	0
5	0.264	0.94	1 (F)	0.87	0
6	0.528	0.98	0	0.85	0
7	1.056	0.42	2 (M,[b] m)	0.88	1 (m)
8	1.584	0.24	1 (M)	0.31	1 (m)
9	2.112	0.13	0	0.34	1 (m)
10	2.640	0.13	0	0.21	0
11	3.168	0.12	0	0.18	1 (M)
12	4.224	0.12	0	0.17	0
13	5.280	0.11	1 (m)	0.18	0
14	6.336	0.12	0	0.18	0
15	8.448	0.07	0	0.20	1 (m)
16	16.896	—	—	0.19	0
Total (all doses)		11.00	11	10.00	10
Tests for trend		$z = 1.42$ $1P > 0.05$		$z = 0.61$ Not significant	

[a] See footnotes to Table 12.
[b] Ureter (N.B.: No urethral neoplasms were reported).

Table 19 *Observed and expected numbers of animals with tumors of the ovary, seminal vessels, etc. (including prostate)*

Treatment group	Concentration (ppm)	Control and NDEA		Control and NDMA	
		Expected	Observed[a]	Expected	Observed[a]
		A. Females			
1	0	3.67	4 (3F, f)	4.32	4 (3F, f)
2	0.033	0.89	0	1.40	2 (2F)
3	0.066	0.74	0	1.31	2 (F, f)
4	0.132	0.47	1 (f)	0.88	0
5	0.264	1.17	1 (f)	1.19	1 (F)
6	0.528	0.41	1 (f)	0.84	0
7	1.056	0.21	0	1.14	2 (2f)
8	1.584	0.13	0	0.41	0
9	2.112	0.06	0	0.31	1 (f)
10	2.640	0.07	0	0.13	0
11	3.168	0.06	0	0.06	0
12	4.224	0.05	0	0.01	0
13	5.280	0.03	1 (f)	—	—
14	6.336	0.02	0	—	—
15	8.448	—	—	—	—
16	16.896	—	—	—	—
Total (all doses)		8.00	8	12.00	12
Tests for trend		$z = 2.28$ $1P = 3.8\%$		$z = 0.29$ Not significant	
		B. Males			
1	0	1.15	0	2.44	0
2	0.033	0.29	0	0.72	0
3	0.066	0.33	1 (M)	0.63	1 (m)
4	0.132	0.34	0	0.65	0
5	0.264	0.24	0	0.59	0
6	0.528	0.34	0	0.73	1 (m)
7	1.056	0.24	2 (M, M[b])	0.53	2 (M, M[c])
8	1.584	0.06	0	0.44	0
9	2.112	0.02	0	0.56	1 (m)
10	2.640	—	—	0.26	1 (M)
11	3.168	—	—	0.20	1 (M)
12	4.224	—	—	0.15	0
13	5.280	—	—	0.06	1 (M)
14	6.336	—	—	0.04	0
15	8.448	—	—	0.01	0
16	16.896	—	—	—	—
Total (all doses)		3.00	3	8.00	8
Tests for trend		$z = 2.21$ $1P = 4.0\%$		$z = 3.21$ $1P = 0.4\%$	

[a] See footnotes to Table 12.
[b] Prostate.
[c] Cowper's complex.

of the lower jaw (and, perhaps, the stomach) in relation to NDEA, inasmuch as the cell types involved are rather similar to those involved in the effects of NDEA on the esophagus; moreover, this agent can undoubtedly cause stomach tumors in mice. Finally, *N*-nitrosopiperidine, another nitrosamine that, like NDEA, strongly affects the esophagus, definitely causes tumors of the lower jaw in Colworth rats.[18]

3. Tumors of the Esophagus or Liver at Low Dose Rates

3A. General Principles of Dose-Response Relationships at Low Dose Rates. There have been several theoretical investigations of the likely shapes of dose-response relationships at low dose rates [for review, see Armitage (5)]. Some of these investigations have inappropriately ignored the possibility that a "background" exists of cellular lesions that produce "spontaneous" neoplasms by cellular mechanisms analogous to the mechanisms whereby the test agent produces cancer. The theoretical investigations that have not explicitly or implicitly ignored this possibility have concluded that, at dose rates so low that the number of treatment-induced neoplasms does not greatly exceed the number of such spontaneous neoplasms, two simple rules are likely to govern the dose-response relationship.

First, there is likely to be no material dependence on dose rate of the distribution of the ages at which treatment-induced tumors arise. Particularly, the strong dependence on dose rate of mean age at onset of cancer that is seen at high dose rates will effectively cease, so that in the low dose range varying the dose rate may importantly affect the number of individuals that develop cancer, but is not likely to have much effect on the ages at disease onset of those individuals in whom treatment induces cancer.

Second, the excess risk of cancer is likely to be approximately proportional to the dose-rate.[19]

It is difficult to illustrate these general rules by data from animal experiments, for as soon as the proportion of animals developing tumors falls below 10% or so purely random errors are likely to make the dose-response relationship so irregular that its shape is difficult to be sure of. However, epidemiologists have observed thousands of lung cancers among hundreds of thousands of smokers, and these rules are illustrated by the observation that, among men who have smoked cigarettes throughout adult life, the absolute risk of lung cancer is approximately proportional to the number smoked per day but the mean age at onset of the disease is virtually unrelated to the daily dose rate. (Although cigarette smoke exposure accounts for about one-third of all human cancer deaths it is, by the standards of animal experiments, a weak carcinogen, for even among heavy smokers only a minority die of lung cancer.)

In the theoretical investigations that gave rise to these general principles [*e.g.*, Crump *et al.* (6)] about low-dose dose-response relationships, "low" had to be defined as producing a carcinogenic effect that did not greatly exceed the number of "spontaneous" neoplasms arising by background exposure to analogous cellular mechanisms. This means that if this background incidence is *appreciable* then a low dose range should exist in which simple proportionality between dose and effect is actually observable, but that if this background incidence is *negligible* then no general prediction follows of the shape of the dose-response relationship.

3B. Implications and Findings. In the present experiment, the background incidence of liver neoplasms is appreciable (involving 29 control animals) but that of esophageal neoplasms is not; no control or NDMA-treated animal was found to have any neoplastic, or even hyperplastic, lesion of the esophagus. Consequently, it may be reasonable tentatively to predict linearity of effect in the low dose range only for liver neoplasms, and not for esophageal neoplasms. Fig. 13 indicates that, in the dose range from 0 to 1 ppm of nitrosamines, the data are consistent with either a linear or a nonlinear response. There is significant upward curvature in the dose response for esophageal neoplasms but not in that for liver neoplasms. The choice of which dose range to study in order to characterize the low-dose effects was dictated by Fig. 1, which showed that the overall pattern of longevity (and hence of extent of exposure to risk of disease onset) was similar for groups 1–7 but not for groups 8 and above.

[18] Unpublished data.

[19] Strictly, these rules are likely to govern the relationship between the carcinogenic effect and the effective dose rate at the intracellular target. At dose rates sufficiently low for no enzyme system to be strongly induced and for no enzyme system to be nearly saturated this is likely in turn to be approximately proportional to the dose rate administered to the whole animal.

In Fig. 13, data have been pooled for both males and females, and for both NDMA and NDEA, in order to obtain group sizes sufficiently large for the proportion of affected animals to be estimated reasonably reliably. By pooling both sexes and both agents, a total of 240 animals at each dose level results, 48 of which were scheduled to be sacrificed after 12 or 18 months of treatment. (The numbers of affected animals in Fig. 13 are therefore what might be expected among about 200 animals that were not scheduled for premature sacrifice and that had a survival pattern like that of the control groups in Fig. 1.)

However, although such a process of pooling confers statistical stability, it does so at the expense of a considerable degree of artificiality. First, if males and females are to be pooled then it is not clear whether this should be on the basis of mg/kg/day rather than ppm v/v (see "Methods," Section 8), while if NDEA and NDMA are to be pooled then perhaps this should be done on the basis of equimolar doses. These problems, however, affect each point rather similarly and thus should tend to cancel out in assessing whether the various dose-response relationships are approximately linear. Second and far more important, it would clearly be preferable to know the dose-response relationships separately for NDEA-treated males, NDEA-treated females, NDMA-treated males, and NDMA-treated females and, to know each separately for each separate type of neoplastic or nonneoplastic lesion rather than, as in Fig. 13, for "all liver neoplasms" (or for "all dose-related liver lesions"). However, examination of the total numbers of affected animals at the lowest dose levels (*e.g.*, below 0.3 ppm) shows that even in the pooled analysis only about 20 liver tumor-bearing animals were observed at each dose level, so only about five would be observed in one sex on one nitrosamine, and even these would not be all of the same histological type. Consequently, although some of the specific dose-response relationships are presented in Tables 21–27, if each were examined separately, so few animals would be affected that it would be impossible to characterize reliably the shapes of any of these separate dose-response relationships below a dose of about 1 ppm. By contrast, with the pooled analysis in Fig. 13 an apparent increase in liver lesions is produced by a dose of only 0.3 ppm, and even among the four

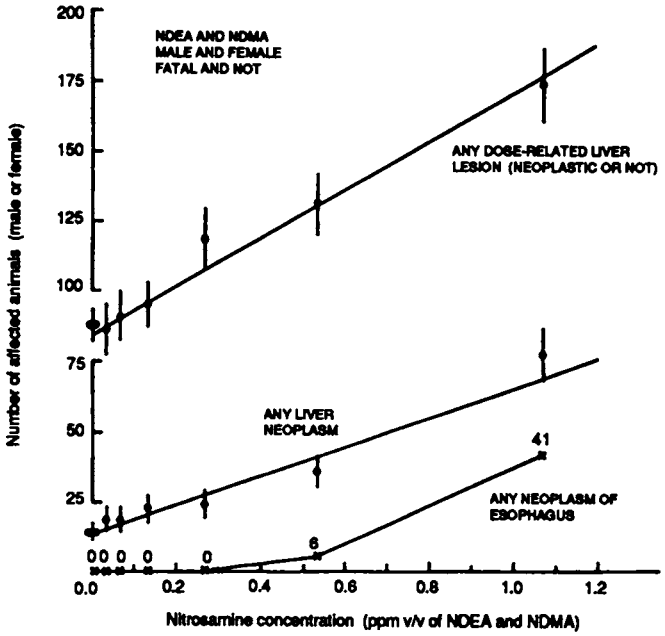


Fig. 13. Pooled dose-response relationships in the low dose range. The "pool" is of both sexes, and of both NDEA and NDMA. ×, esophageal lesions; ●, liver lesions. *Bars*, ranges of rates 1 SD above and below observed rates. Each treated group (but not the controls) began with 240 animals (60 male NDEA, 60 female NDEA, 60 male NDMA, 60 female NDMA), of which 192 were not scheduled for sacrifice at 12 or 18 months. The control group was double this size, so the control numbers are halved and plotted twice.

Table 20 *Observed and expected numbers of animals with lymphatic or hematopoietic neoplasms, presented separately for (a) males and (b) females because of the (apparent) discrepancy between the responses of the two sexes to NDMA*

Note: The diagnosis of such neoplasms was generally based chiefly on the blood that was fixed in samples from the lung or other tissues and this was not as reliable as if it were based on standard blood smears. (There is, however, no reason to expect it to be particularly biased in NDMA-treated females.)

Treatment group	Concentration (ppm)	Control and NDEA, males		Control and NDEA, females		Control and NDMA, males		Control and NDMA, females	
		Expected	Observed[a]	Expected	Observed[a]	Expected	Observed[a]	Expected	Observed[a]
1	0	20.11	22 (17M, 5m)	10.25	10 (9F, f)	14.33	22 (17M, 5m)	10.59	10 (3F, 6F[b], f[b])
2	0.033	4.69	5 (3M, 2m)	2.47	1 (F)	4.14	1 (M)	3.54	1 (f[b])
3	0.066	5.72	5 (3M, 2m)	2.38	3 (2F, f)	3.65	6 (5M, m)	2.94	2 (F, F[b])
4	0.132	6.15	5 (2M, 3m)	1.87	5 (5F)	3.78	1 (M)	2.27	2 (F, F[b])
5	0.264	4.62	1 (M)	3.06	2 (2F)	3.54	1 (M)	2.87	0
6	0.528	5.61	4 (4M)	1.56	1 (F)	4.22	4 (2M, 2m)	1.70	4 (F, 2F[b], f[b])
7	1.056	3.88	6 (5M, m)	1.07	2 (2F)	3.20	2 (M, m)	2.60	7 (F, 5F[b], f[b])
8	1.584	2.35	1 (m)	0.65	0	2.87	3 (3m)	0.98	1 (F[b])
9	2.112	1.24	3 (3M)	0.24	0	3.19	2 (M, m)	0.89	0
10	2.640	1.02	1 (M)	0.14	0	1.72	1 (M)	0.47	1 (f[b])
11	3.168	0.81	1 (M)	0.12	0	1.44	0	0.38	0
12	4.224	0.59	3 (3M)	0.08	0	1.05	5 (5M)	0.27	0
13	5.280	0.43	1 (M)	0.06	0	0.35	0	0.18	0
14	6.336	0.35	0	0.03	0	0.30	0	0.18	1 (F)
15	8.448	0.31	0	0.01	0	0.17	0	0.09	0
16	16.896	0.10	0	—	—	0.06	0	0.04	1 (F)
Total (all doses)		58.00	58 (44M, 14m)	24.00	24 (22F, 2f)	48.00	48 (35M, 13m)	30.00	30 (25F, 5f)
Sex-specific trends:		$z = 1.01$ Not significant		$z = -0.87$ Not significant		$z = -0.07$ Not significant		$z = 3.74$ $1P \simeq 0.3\%$	
Pooled test for trend:		$z = 0.71$ Not significant				$z = 2.00$ $1P \simeq 3.2\%$			

[a] See footnotes to Table 12.
[b] Tentatively specified as having arisen from the lymph nodes or spleen. (The remaining neoplasms among the NDMA-treated females were specified merely as being "reticuloendothelial".)

Table 21 *Numbers of low-dosed animals with liver cell neoplasm reported*

N.B.: Most were malignant; for details, see Tables 6 and 7.

Treatment group	Nitrosamine concentration (ppm)	NDEA			NDMA			NDEA and NDMA		
		M	F	M and F	M	F	M and F	M	F	M and F
1	0	10/4[a] =2.5	11/4[a] =2.75	21/4[a] =5.25	10/4[a] =2.5	11/4[a] =2.75	21/4[a] =5.25	10/2[a] =5.0	11/2[a] =5.5	21/2[a] =10.5
2	0.033	1	4	5	4	2	6	5	6	11
3	0.066	2	4	6	3	2	5	5	6	11
4	0.132	3	3	6	2	4	6	5	7	12
5	0.264	0	4	4	4	2	6	4	6	10
6	0.528	5	10	15	4	6	10	9	16	25
7	1.056	9	31	40	5	6	11	14	37	51
Initial[b] No./ treated group		60	60	120	60	60	120	120	120	240

[a] The control group consisted of 240 of each sex initially, *i.e.*, 4 times as many as the NDEA- or NDMA-treated groups, and 2 times as many as their pool.
[b] The effective group size was somewhat smaller than indicated due to the scheduled sacrifices at 12 and at 18 months.

Table 22 *Numbers of low-dosed animals with bile duct neoplasm reported*

N.B.: Most were benign; for details, see Tables 6 and 7.

Treatment group	Nitrosamine concentration (ppm)	NDEA			NDMA			NDEA and NDMA		
		M	F	M and F	M	F	M and F	M	F	M and F
1	0	3/4[a] =0.75	4/4[a] =1.0	7/4[a] =1.75	3/4[a] =0.75	4/4[a] =1.0	7/4[a] =1.75	3/2[a] =1.5	4/2[a] =2.0	7/2[a] =3.5
2	0.033	2	2	4	2	1	3	4	3	7
3	0.066	0	0	0	3	4	7	3	4	7
4	0.132	1	1	2	2	1	3	3	2	5
5	0.264	2	2	4	2	4	6	4	6	10
6	0.528	0	1	1	1	4	5	1	5	6
7	1.056	4	2	6	1	9	10	5	11	16
Initial[b] no./ treated group		60	60	120	60	60	120	120	120	240

[a] The control group consisted of 240 of each sex initially, *i.e.*, 4 times as many as the NDEA- or NDMA-treated groups, and 2 times as many as their pool.
[b] The effective group size was somewhat smaller than indicated due to the scheduled sacrifices at 12 and at 18 months.

Table 23 *Numbers of low-dosed animals with mesenchymal hepatoma reported*

N.B.: Most were malignant; for details, see Tables 6 and 7.

Treatment group	Nitrosamine concentration (ppm)	NDEA			NDMA			NDEA and NDMA		
		M	F	M and F	M	F	M and F	M	F	M and F
1	0	0/4[a] =0.0	0/4[a] =0.0	0/4[a] =0.0	0/4[a] =0.0	0/4[a] =0.0	0/4[a] =0.0	0/2[a] =0.0	0/2[a] =0.0	0/2[a] =0.0
2	0.033	1	0	1	0	1	1	1	1	2
3	0.066	0	0	0	0	0	0	0	0	0
4	0.132	4	0	4	1	0	1	5	0	5
5	0.264	1	0	1	1	1	2	2	1	3
6	0.528	3	1	4	0	1	1	3	2	5
7	1.056	3	1	4	1	2	3	4	3	7
Initial[b] no./ treated group		60	60	120	60	60	120	120	120	240

[a] The control group consisted of 240 of each sex initially, *i.e.*, 4 times as many as the NDEA- or NDMA-treated groups, and 2 times as many as their pool.
[b] The effective group size was somewhat smaller than indicated due to the scheduled sacrifices at 12 and at 18 months.

lowest dose levels (of zero, 0.033, 0.066, and 0.132 ppm) a positive trend in the proportions of animals with liver cancer is apparent that just attains statistical significance ($1P = 1.7\%$; see Table 28). Although such pooling may not be totally appropriate, examination of the pooled dose response allows observation of patterns that might be drowned in random error in each of the several separate dose responses.

Note that, to determine whether or not a particular low dose level has any carcinogenic effect, a standard significance test (either for trend with respect to this and lower doses or for a difference between this and the control group) may not be an appropriate statistical procedure, for if, as for liver neoplasms, there is an approximately linear trend at 0.5 and 1.0 ppm and no suggestion of any divergence from this linear trend at any lower dose then the general theoretical arguments (5–7) that lead one to expect linearity at low doses when the background incidence is appreciable become even stronger. Such data therefore provide some evidence that, *throughout* the dose range below 1 ppm (*i.e.*, not only in the restricted range 0.1–1 ppm suggested by the significance tests) there is approximate proportionality of excess risk to applied dose rate. For the reasons already discussed, we may take the effective denominator to be about 200 rats exposed lifelong at each treatment level. The slope of the line through the data for liver neoplasms in Fig. 13 then suggests that a dose of 1 ppm will cause about 25% of such rats to develop liver tumors, that a dose of 0.1 ppm will cause about 2.5% to do so, and (although this could not be observed directly in any experiment of realistic size) that a dose

Table 24 *Numbers of animals with any liver neoplasm reported*

For histological details, see Tables 6 and 7.

Treatment group	Nitrosamine concentration (ppm)	NDEA			NDMA			NDEA and NDMA		
		M	F	M and F	M	F	M and F	M	F	M and F
1	0	13/4[a] =3.25	16/4[a] =4.0	29/4[a] =7.2	13/4[a] =3.25	16/4[a] =4.0	29/4[a] =7.2	13/2[a] =6.5	16/2[a] =8.0	29/2[a] =14.5
2	0.033	4	6	10	5	4	9	9	10	19
3	0.066	2	4	6	7	6	13	9	10	19
4	0.132	9	4	13	5	5	10	14	9	23
5	0.264	4	7	11	6	7	13	10	14	24
6	0.528	7	12	19	5	12	17	12	24	36
7	1.056	15	35	50	9	18	27	24	53	77
Initial[b] no./ treated group		60	60	120	60	60	120	120	120	240

[a] The control group consisted of 240 of each sex initially, *i.e.*, 4 times as many as the NDEA- or NDMA-treated groups, and 2 times as many as their pool.
[b] The effective group size was somewhat smaller than indicated due to the scheduled sacrifices at 12 and at 18 months.

Table 25 *Numbers of animals with hyperplastic nodules but no liver neoplasm reported*

Treatment group	Nitrosamine concentration (ppm)	NDEA			NDMA			NDEA and NDMA		
		M	F	M and F	M	F	M and F	M	F	M and F
1	0	4/4[a] =1.0	14/4[a] =3.5	18/4[a] =4.5	4/4[a] =1.0	14/4[a] =3.5	18/4[a] =4.5	4/2[a] =2.0	14/2[a] =7.0	18/2[a] =9.0
2	0.033	2	3	5	4	8	12	6	11	17
3	0.066	2	6	8	4	3	7	6	9	15
4	0.132	3	1	4	1	3	4	4	4	8
5	0.264	1	9	10	1	4	5	2	13	15
6	0.528	4	8	12	3	4	7	7	12	19
7	1.056				Nos. at risk reduced by hepatomas					
Initial[b] no./ treated group		60	60	120	60	60	120	120	120	240

[a] The control group consisted of 240 of each sex initially, *i.e.*, 4 times as many as the NDEA- or NDMA-treated groups, and 2 times as many as their pool.
[b] The effective group size was somewhat smaller than indicated due to the scheduled sacrifices at 12 and at 18 months.

Table 26 *Numbers of animals with shrinkage of hepatocytes but no liver neoplasm reported*

Treatment group	Nitrosamine concentration (ppm)	NDEA			NDMA			NDEA and NDMA		
		M	F	M and F	M	F	M and F	M	F	M and F
1	0	42/4[a] =10.5	36/4[a] =9.0	78/4[a] =19.5	42/4[a] =10.5	36/4[a] =9.0	78/4[a] =19.5	42/2[a] =21.0	36/2[a] =18.0	78/2[a] =39.0
2	0.033	8	7	15	10	13	23	18	20	38
3	0.066	10	11	21	9	10	19	19	21	40
4	0.132	13	14	27	12	12	24	25	26	51
5	0.264	24	16	40	10	8	18	34	24	58
6	0.528	23	9	32	13	9	22	36	18	54
7	1.056				Nos. at risk reduced by hepatomas					
Initial[b] no./ treated group		60	60	120	60	60	120	120	120	240

[a] The control group consisted of 240 of each sex initially, *i.e.*, 4 times as many as the NDEA- or NDMA-treated groups, and 2 times as many as their pool.
[b] The effective group size was somewhat smaller than indicated due to the scheduled sacrifices at 12 and at 18 months.

Table 27 *Numbers of animals with any neoplastic or dose-related non-neoplastic liver lesion reported*

For list of nonneoplastic dose-related lesions, see Table 8.

Treatment group	Nitrosamine concentration (ppm)	NDEA			NDMA			NDEA and NDMA		
		M	F	M and F	M	F	M and F	M	F	M and F
1	0	95/4[a] =23.75	80/4[a] =20.0	175/4[a] =43.75	95/4[a] =23.75	80/4[a] =20.0	175/4[a] =43.75	95/2[a] =47.5	80/2[a] =40.0	175/2[a] =87.5
2	0.033	22	17	39	23	24	47	45	41	86
3	0.066	20	22	42	24	25	49	44	47	91
4	0.132	29	24	53	19	23	42	48	47	95
5	0.264	34	34	68	28	23	51	62	57	119
6	0.528	40	33	73	27	31	58	67	64	131
7	1.056	45	51	96	37	40	77	82	91	173
Initial[b] no./ treated group		60	60	120	60	60	120	120	120	240

[a] The control group consisted of 240 of each sex initially, *i.e.*, times as many as the NDEA- or NDMA-treated groups, and 2 times as many as their pool.
[b] The effective group size was somewhat smaller than indicated due to the scheduled sacrifices at 12 and at 18 months.

Table 28 *Pooled trend in four lowest dose groups*

This table gives an analysis, stratified for sex, of the numbers of liver tumor-bearing animals, pooling the NDEA-treated and the NDMA-treated animals at each dose level. It is obtained by combining a death rate analysis of the fatal liver tumors and a prevalence analysis of the incidental liver tumors. N.B.: At these four dose levels no animal was found to have any hyperplastic or neoplastic lesions of the esophagus, and no liver neoplasms were found at the scheduled sacrifices at 12 or at 18 months.

Treatment group	Nitrosamine concentration (NDEA/NDMA) (ppm)	Initial group size	No. not scheduled for sacrifice	Nos. with any liver neoplasm		
				(a) Observed (*O*)	(b) Expected (*E*)	(c) Ratio (*O*/*E*)
1	0	480	384	29	35.9	0.81
2	0.033	240	192	19	19.6	0.97
3	0.066	240	192	19	18.1	1.05
4	0.132	240	192	23	16.4	1.40
Total (all doses)		1200	960	90	90	1.00

Test for trend: $T = 0.091$, $z = 2.16$, $1P = 1.7\%$.

of 0.01 ppm could cause up to 0.25% to do so. The general rule appears thus to be that the effects of low doses on liver tumors greatly exceed their effects on esophageal tumors and that the lifelong excess risk of rat liver tumor induction at a given nitrosamine concentration is approximately that obtained by multiplying the concentration in ppm by a figure of 25% (so 0.1 ppm yields a 2.5% excess risk, etc.).

This provides us with what is probably a reasonably reliable estimate (despite the practical impossibility of direct confirmation) of the effects of ppb nitrosamine concentrations *on rats* under these experimental circumstances, but it does not provide reliable information as the effects of ppb nitrosamine concentrations on ***humans***, and it would be a serious distortion of these experimental results to suggest otherwise. [Some reasons for this are discussed by Peto (8) and Doll and Peto (Ref. 9; Section 4.2)].

There has been no attempt in the present report to connect the low-dose dose response relationship with the high-dose dose response relationship, because the former has been analyzed in terms of numbers of affected animals, while the latter was analyzed in terms of median time to death from each disease of interest, and a unified analysis of the two will come only when, in a separate report, each is analyzed in terms of Weibull distributions.

Discussion

The principal carcinogenic effects that were studied in this experiment were of NDEA on the esophagus and hepatocytes and of NDMA on the bile ducts and hepatocytes.

Lesser effects were also demonstrated of both agents on mesenchymal and Kupffer cells in the liver, of NDMA on the lung, and of NDEA on the nasopharynx and bile ducts. Finally, although some further associations with NDEA or NDMA were noted that were suggestive of effects on certain other sites, none was entirely convincingly demonstrated in this experiment.

The four dose-response relationships for the four principal carcinogenic effects were studied separately in the high dose range where treatment caused substantial numbers of deaths from each disease. For each of the four, the effect of a given duration of treatment on females exceeded that on males. For each, an approximate straight-line relationship was observed between the dose rate and the median time to death just from the disease of interest. Three of these four main dose-response relationships (NDMA-induced bile duct neoplasms, NDEA-induced esophageal neoplasms, and NDEA-induced hepatocellular neoplasms) had a similar slope of about -1 in about 2.3. This slope indicates that approximately a 5-fold increase in dose rate is needed to halve the median time to death from the disease of interest. The fourth, however (NDMA-induced hepatocellular neoplasms), appeared to have a much steeper slope, of about -1, which indicates that only a 2-fold increase in dose rate is needed to halve the corresponding median. The reasons for this difference in slope are at present obscure.

The slope of -1 in about 2.3 observed for three of the four relationships corresponds exactly with the observation by Druckrey (10) that his experimental findings (for NDEA in rat liver) obeyed the relationship

$$(\text{dose rate}) \times (\text{median})^{2.3} = \text{constant}$$

even though the experimental conditions were different, the anatomic sites were not all the same, and the detailed definition of the median time-to-tumor differed. Whether or not Druckrey's relationship holds exactly for three of our four main dose-response relationships, it clearly holds approximately throughout the high-dose range (although not necessarily outside this range). Also, even though the proper exponent for such data may not be exactly 2.3, it must be approximately this, although values of 3 or 2 may still not be excluded.

Such mathematical relationships are sometimes taken as evidence that deaths from these neoplasms have a "lognormal" distribution (with the mean log-time-to-tumor related to the dose-rate, as in Figs. 4, 10, or 11), but this is not justifiable. Not only is there little reason to expect a "lognormal" distribution to govern such data (11) but also precisely this form of mathematical relationship could be produced by other distributions such as the "Weibull" distributions [with constant "shape" parameters (4)], which have been particularly recommended for the description of tumor onset times in continuous carcinogenesis studies such as the present experiment.[20]

Of course, in reality no single simple mathematical formula is going to provide an absolutely precise fit to such data. This may not matter much while attention is restricted to a range of high doses that has been studied directly, for this is the range of doses to the effects of which the chosen formula will have been fitted without any serious discrepancy. It may, however, matter enormously if any attempt is to be made to extrapolate the formula out of the dose range where it has been at least approximately verified and down to far lower doses, for at low

[20] A straight line with slope -1 in 2.3 (as in Figs. 4, 10*AB*, and 11*CD*) would be predicted, for example, by a Weibull distribution in which after t weeks of treatment with a dose rate d of nitrosamine the probability per survivor of death tomorrow from the tumor type of interest is proportional to d^3t^6.

doses there may be large differences between the predictions of two different formulae that both fit the high-dose data reasonably well. Recourse may then have to be made to general theories about the likely nature of low-dose dose-response relationships [for review, see Armitage (5)]. The appropriate ones of these predict that *if* there is an appreciable background of "spontaneous" neoplasms of whatever type is of interest, *then* the dose-response relationship is likely to be simply linear at dose rates so low that the induced risk does not greatly exceed the background risk (with the age distribution of any tumors that are induced being virtually independent of the dose rate).

No general predictions can be made of the shape of the dose-response relationship at low doses if the spontaneous rate is immeasurably small, as for esophageal neoplasms in the present study. Consequently, it is unsurprising to note that at low doses the onset rate of esophageal cancer appears not to be simply proportional to dose.

For liver cancer, however, the background rates are about 8%, which is appreciable. As might be expected in view of this, in the range of nitrosamine concentrations below 1 ppm in the water, the excess risk of liver cancer appears to be roughly proportional to the nitrosamine concentration (with a 25% excess at about 1 ppm, a 2.5% excess at about 0.1 ppm, etc.). Since the relationship has been observed to be approximately linear in the range 0.1–1 ppm, the general arguments about the likely shapes of dose-response relationships make it probable that at even lower doses, where direct observation is impracticable, this linear relationship may remain approximately true, for Colworth rats, if not for humans.

Ultimately, it is to be hoped that molecular biological investigations of the transport, activation, action, and repair of effects of nitrosamines will advance to the point where they can predict correctly at least the main qualitative aspects of the dose-response relationships observed in this study. In view of the unique size of the present study, this process may be facilitated if, in selecting a strain of rats for some detailed laboratory investigation of the mechanisms of action of nitrosamines, research workers henceforth adopt a policy of studying inbred Colworth rats unless there are strong reasons to do otherwise.

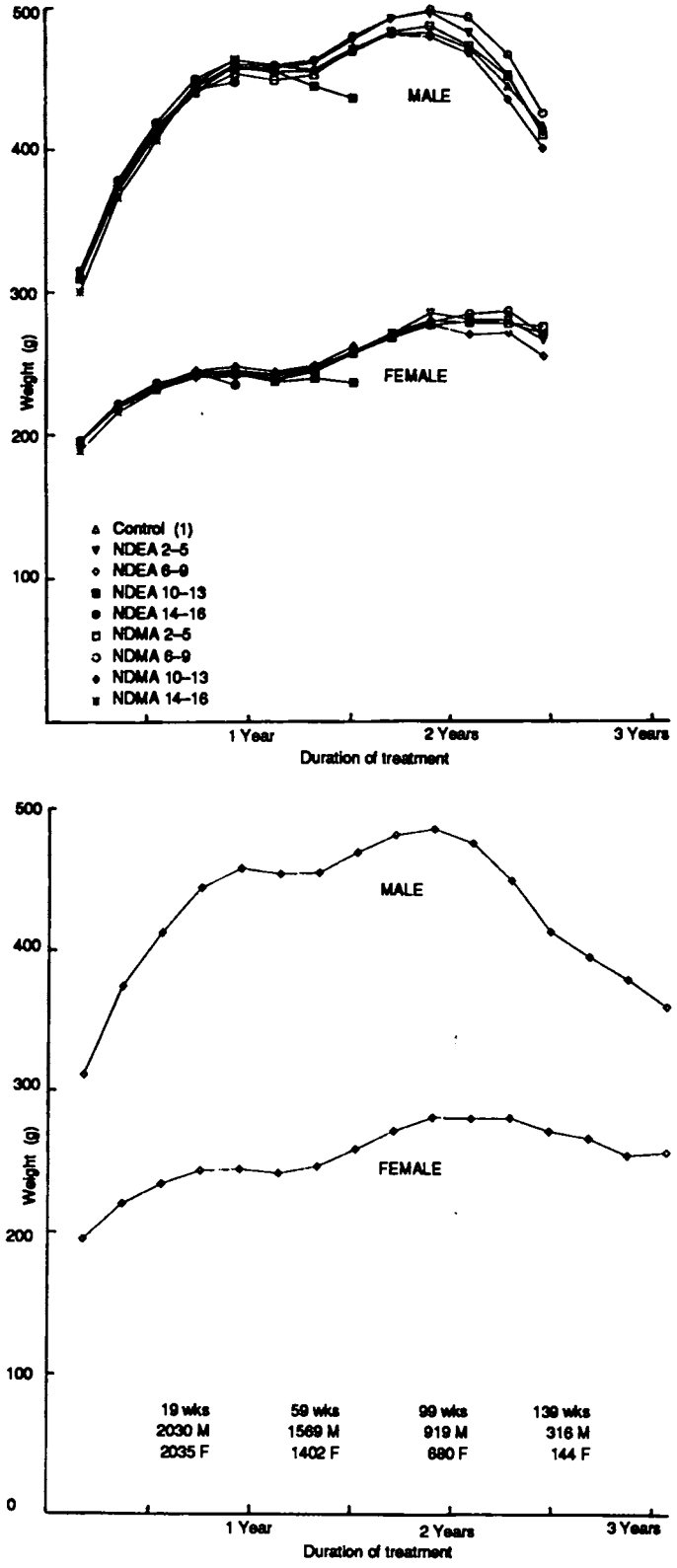


Fig. A. *Top*, Mean body weight per survivor, by treatment group and sex. Animals were weighed weekly, but data were key-punched only for weeks 9, 19, 29, etc. *Bottom*, mean body weight per survivor by sex.

Appendix 1: Allowance for the Possible Nonnormality of the Trend Test Statistic in Estimation of 1-tailed *P* Values

As is commonly the case in animal experiments, the absolute differences between successive dose levels are smaller in the low-dose range than in the high-dose range, so the distribution of dose levels is positively skewed. The combined effects of the positive skewness of the dose levels tested and the early mortality of the high-dose groups (from tumors of the liver and/or esophagus) together imply that the test statistic for positive trend will, for all types of lesion, have a statistical distribution that is also positively skewed, even under the "null hypothesis" that the age-specific onset rates of the lesions of interest are unaffected by treatment. The 1-tailed *P* values estimated from a normal approximation to this "null hypothesis" distribution of the trend statistic may therefore be somewhat too extreme. This means that if the test statistic for positive trend, *T*, is less than 1.96 of its standard errors above zero, the trend is certainly not conventionally significant ($1P > 0.025$) but that if *T* only moderately exceeds two of its standard errors the normal approximation may not be of adequate accuracy. More accurate estimation of the 1-tailed statistical significance level, 1*P*, may then be helpful. This may be achieved via calculation of all of the first four moments (zero, $M2$, $M3$, and $M4$) of the null hypothesis distribution of *T*. Then, 1*P* can be estimated by an analogy between the null hypothesis distribution of *T* and a standard statistical distribution with not only the same mean (zero) and variance ($M2$), but also the same third ($M3$) and fourth ($M4$) moments as the null hypothesis distribution of *T*. An appropriate Johnson distribution, selected as described by Hill *et al.* (12), has been used for this purpose.

Calculation of First Four Moments of *T*(0, $M2$, $M3$, and $M4$). This calculation depends on the fact that in the present experiment, with 16

dose levels $d_1, d_2, \ldots, d_{16}$ (d_1 being zero, since group 1 is the control group), the quantity T is derived, as described in Ref. 3, by accumulation of one quantity from each of several separate 2 × 16 tables relating the presence or absence of disease (at a particular time or range of times) to group membership. For one such "simple" contingency table, let the notation of Appendix Fig. 1 (*below*) be adopted: m_1 denotes the number of animals in group 1 currently at risk; m_2 denotes the number of animals in group 2 currently at risk; and so on; m_{16} denotes the number of animals in group 16 currently at risk.

Appendix Fig. 1. Notation for a single simple (multivariate hypergeometric 2 × 16 table.

Dose level	d_1	d_2	...	d_{16}	All doses
No. with lesion	o_1	o_2	...	o_{16}	n
			...		
No. at risk	m_1	m_2	...	m_{16}	M

M denotes the total number of animals (so $M = m_1 + m_2 + \ldots + m_{16}$), n denotes the number of these that have the disease of interest (which, in the case of tumors, would be the total number of tumor-bearing animals, not the total number of tumors), and $\bar{d}$ denotes the average of the doses administered to the animals in this particular table, so

$$\bar{d} = d_1m_1/M + d_2m_2/M + \ldots + d_{16}m_{16}/M$$

Now, define

$$S_2 = (d_1 - \bar{d})^2m_1 + (d_2 - \bar{d})^2m_2 + \ldots + (d_{16} - \bar{d})^2m_{16}$$

$$S_3 = (d_1 - \bar{d})^3m_1 + (d_2 - \bar{d})^3m_2 + \ldots + (d_{16} - \bar{d})^3m_{16}$$

$$S_4 = (d_1 - \bar{d})^4m_1 + (d_2 - \bar{d})^4m_2 + \ldots + (d_{16} - \bar{d})^4m_{16}$$

$$p_1 = n/M$$

$$p_2 = n(n-1)/[M(M-1)] \quad \text{(or zero if } n \le 1\text{)}$$

$$p_3 = n(n-1)(n-2)/[M(M-1)(M-2)] \quad \text{(or zero if } n \le 2\text{)}$$

$$p_4 = n(n-1)(n-2)(n-3)/[M(M-1)(M-2)(M-3)] \quad \text{(or zero if } n \le 3\text{)}$$

Let o_1 denote the number of animals with the disease of interest in group 1, let o_2 denote the number of animals with the disease of interest in group 2, ..., and let o_{16} denote the number of animals with the disease of interest in group 16.

If we define $t = o_1(d_1 - \bar{d}) + o_2(d_2 - \bar{d}) + \ldots + o_{16}(d_{16} - \bar{d})$, then it can be shown[21] that, under the "null hypothesis" that treatment does not affect the onset rate of disease among animals of a given age, the first four moments of t are, respectively, zero, μ_2, μ_3, and μ_4, where

$$\mu_2 = (p_1 - p_2)S_2 \quad \text{(N.B. } \mu_2 = \text{variance of } t\text{)}$$

$$\mu_3 = (p_1 - 3p_2 + 2p_3)S_3$$

$$\mu_4 = (p_1 - 7p_2 + 12p_3 - 6p_4)S_4 + 3(p_2 - 2p_3 + p_4)S_2^2$$

For a "normal" distribution, the fourth moment is equal to 3 times the square of the variance, but the distribution of t may not be normal so, if we define the quantity κ_4 to be $\mu_4 - 3\mu_2^2$, κ_4 may differ from zero. (κ_4 is, in statistical terminology, called the "fourth cumulant" of the distribution of t under the null hypothesis.)

In the IARC report (3) it is shown that (again under the "null hypothesis") these values of t (one from each such 2 × 16 table) behave as statistically independent random variables and that their sum is T, the overall trend test statistic. Consequently, if we let $K4$ denote $M4 - 3(M2)^2$ (*i.e.*, the amount by which the fourth moment of T exceeds the square of its variance), it follows (by standard statistical arguments about cumulants) that: T = sum of separate t values, one from each separate 2 × 16 table; and $M2$ = sum of μ_2 values, one from each separate 2 × 16 table; and $M3$ = sum of separate μ_3 values, one from each separate 2 × 16 table; and K_4 = sum of separate κ_4 values, one from each separate 2 × 16 table. The use of such arguments to derive M_2 (the variance of T) was described in the IARC report (3), and, as already noted above, this analogous derivation of $M3$ and $K4$ may be helpful because it allows the accuracy of the estimation of the P value by a normal approximation to be checked ($M3$ and $K4$ should be approximately zero if the null hypothesis distribution of T is indeed approximately "normal"), and improved if necessary [either by the use of Johnson distributions, as described by Hill *et al.* (12), or by reference to published tables of critical values of certain nonnormal distributions, *e.g.*, Pearson and Hartley (14), Tables 42–3].

Throughout this report, whether P values were derived by a normal approximation or by some more accurate approximation, no "continuity correction" has been applied for there is not even agreement between statisticians as to whether or not continuity corrections are *desirable*, so they are certainly not *necessary*. (Readers who do desire them so should, however, be able to obtain the necessary information from the details that are tabulated.)

Appendix 2: Control of Random and Systematic Effects of Scheduled Sacrifice, Autolysis, Cannibalism, and Diaphragmatic Hernia

Scheduled Sacrifice. The animals entered in 10 batches, but all treatments were equally represented in each batch, so this caused no bias.[22] Two of these batches underwent sacrifice at two preselected times, while the other 8 were maintained throughout their life span. The possibility cannot be excluded that the degree of care with which small lesions were sought at autopsy might have been systematically different during the scheduled sacrifices (at each of which a few hundred animals were killed within a few days) from that in the routine course of the experiment, and so the statistical methods were modified slightly, in the manner suggested in the IARC report (3), to ensure that no biases could be caused by any such differences in technique.[23]

Total Exclusions from Analyses. The 0.4% of animals that were discovered at autopsy to have the congenital anomaly of a diaphragmatic hernia (which may affect a lobe of the liver) were excluded from all analyses as if they had died on day 1 of the study, as were the 0.8% of animals that were so totally autolyzed or cannibalized postmortem as to preclude any meaningful determination of the presence or absence of major tumors.

Minimization of Bias in Use of Data from Partially Lost Animals for Assessment of Tumor Death Rates. For the 3.0% of animals that were only partially lost postmortem, the presence or absence of many, although not all, types of tumor could be determined at autopsy, and a combination of the experimental records and the material remaining for autopsy provided suggestive evidence that some particular type of tumor had caused that animal's death for 86%, and this evidence was accepted. In the remaining 14% of cases, where this was not so, it was assumed that neoplastic disease had not been an underlying cause of that animal's death. Although this latter assumption must sometimes

[21] *Proof:* The distribution of t is that of the sum of n numbers chosen at random without replacement from a finite population of M numbers, comprising m_1 numbers each with the value $(d_1 - \bar{d})$, m_2 numbers each with the value $(d_2 - \bar{d})$, etc. ..., and m_{16} numbers each with the value $(d_{16} - \bar{d})$. The sums of the first, second, third, and fourth powers of these M numbers are zero, S_2, S_3, and S_4, and the formulae for the first four moments of a sum of n of them are given in Appendix B of Peto and Peto (13).

[22] Moreover, after standardization for the effects of sex and treatment, there was no significant relationship between batch membership (1–10) and either longevity or hepatoma onset rates.

[23] As a particular sacrifice time (batch 1 at month 12 of treatment, batch 2 at month 18 of treatment) all animals of one sex then undergoing scheduled sacrifice were analyzed together, irrespective of the actual day on which sacrifice occurred, since the spread of days was less than 1 week. Data prior to the time of scheduled sacrifice from animals in a batch that was scheduled to be sacrificed contributed normaly to the main analysis of the similar data from all other batches, and the results of this main analysis and of the two analyses of the two scheduled sacrifices were then combined, as described in the IARC report (3).

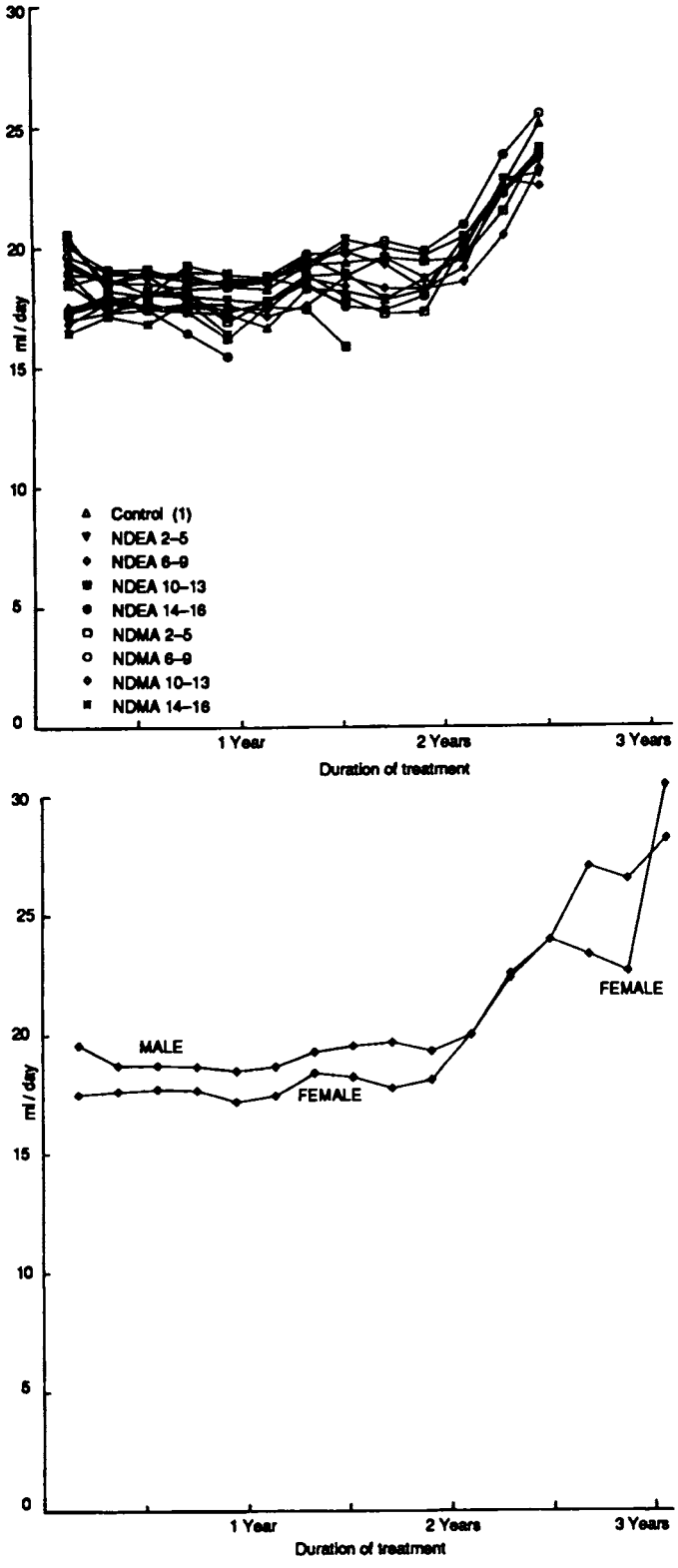


Fig. B. *Top*, mean water intake in ml/survivor, by treatment group and sex. Intake per cage was estimated directly from weekly changes in bottle contents, as spillage and wastage by animals was thought to amount to no more than a few per cent of total usage. *Bottom*, mean daily water intake in ml/rat, by sex.

have been in error, such errors were believed to have been infrequent, and in any case no preferable alternative assumption was apparent.[24]

Minimization of Bias in Use of Data from Partially Lost Animals for Assessment of Tumor Prevalence Rates. Animals that were only partially cannibalized postmortem were categorized in three respects. For the 3.0% of such animals, it was noted: (*a*) whether the head was largely intact (1.4% not); (*b*) whether the esophagus was wholly intact (2.2% not); and (*c*) whether the liver was wholly intact (0.7% not). Anatomic sites were then categorized according to which of these three forms of cannibalism might affect the detectability of small tumors at those sites: for example, the pituitary could be affected only by type *a* cannibalism, while the thyroid could be affected by eitgher type *a* or type *b* cannibalism. Finally, in analyzing the prevalence of incidental tumors at a site that might be affected by certain types of cannibalism, all animals were omitted from both the numerator and the denominator of the prevalence analysis if they had undergone any[25] of that type of cannibalism.

The lack of any significant trends in the last three lines of Table 10AB and of Table 10CD supports the suggestion that no material biases were introduced by the procedures outlined in Appendix 2.

Appendix 3: Laboratory Procedures

Animal Accommodation

The rats were housed in cages constructed from stainless steel and polyethylene. These cages were held in racks, each containing 15 cages. Excreta were collected on high wet strength paper, held on trays beneath the cages.

The animals were kept in four rooms of an animal unit given over specifically to this study. Each room contained one-fourth of the controls (dose level 1) of each sex. Rooms 1 and 2 contained the NDEA-treated rats and rooms 3 and 4 contained the NDMA-treated rats, with the lower dose levels (groups 2–8 plus one-half of group 9) being in rooms 1 and 3.[26] Air was provided to the animal rooms at a rate sufficient to give 15 air changes per hour in each room, filtered to remove particles down to 1 μm in size. Although the air supply unit contained no provision to reduce air temperature below ambient, the temperature of the animal rooms was maintained at 21 ± 2°C, apart from a few isolated occasions when it rose above this in hot weather. The maximum temperature ever reached was 27°C. Humidity was maintained between 50 and 80%. Lighting of the rooms was on a 12-h light, 12-h dark cycle.

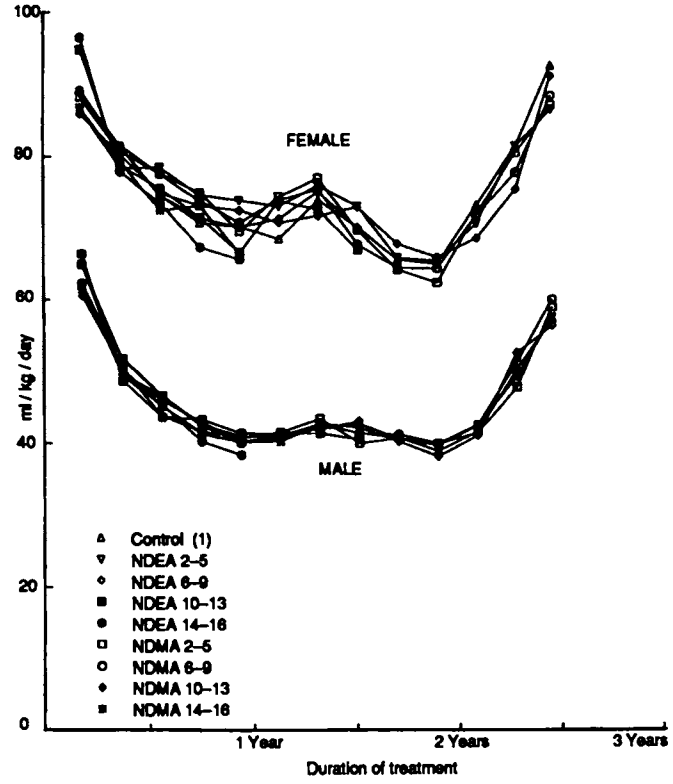
Animal Maintenance Procedures

The paper used for collecting excreta was changed daily. All cages were washed on a 3-weekly rota. When animals were transferred from one cage to another, only one cage was open at any one time. The cages for each treatment group were labeled with waterproof marker to ensure that, even after washing, there was no exchange of cages between

[24] To discard from the analysis only those partially cannibalized animals that appeared not to have died of a tumor would have introduced a serious bias. The choice appeared therefore to be between *either* discarding all the data on tumor death rates from all partially canniblized animals (irrespective of whether or not they appeared to have died of a tumor) *or* making the best guess possible about tumor mortality, as described in the text. Both procedures are potentially slightly biased in the estimates they yield of absolute tumor death rates, the former because cannibalism tends particularly to affect animals that die young and the latter because a few fatal tumors will be overlooked. It was considered that the latter bias was likely to be smaller, and moreover the latter procedure is less wasteful of data than the former. Finally, it should be noted that although the absolute tumor death rates may be slightly biased by the total loss of certain fatal tumors by partial cannibalism, this need not bias the *comparison* of different groups with each other. Thus, it need not alter the probability of obtaining a false positive trend with respect to dose for some particular type of tumor if no material effect of treatment really exists on the age-specific onset rates of such tumors.

[25] Thus, if a large part of the head was missing that animal would not contribute to the analysis of pituitary tumor prevalences even if the pituitary was largely or wholly intact. (Separate categorization of the presence or absence of every possible tissue would have been tedious and unreliable, and in practice not much useful information was lost by utilizing only three broad categories of partial cannibalism in prevalence analyses.)

[26] Cross-contamination is unlikely to have had any material effect on the net nitrosamine exposure of any group, to judge by the fact that hepatomas developed in only 5% of the 240 controls that shared a room with high-dose groups, as opposed to 7.5% of the 240 controls in rooms 1 and 3.



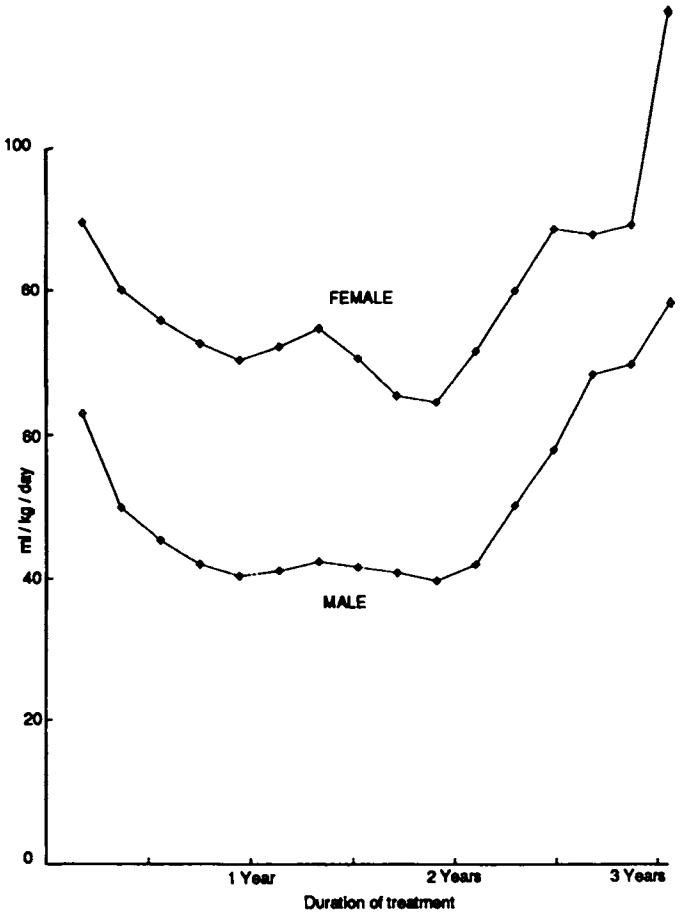


Fig. C. *Top*, mean water intake in ml/kg body weight/day, by treatment group and sex. *Bottom*, mean water intake in ml/kg/day, by sex.

groups. The coding system used was a combination of colors (rooms 1, 2, 3, and 4 being coded as blue, green, red, and yellow) and numbers (0–8 in each room). Thus, for example, a dose of 0.033 ppm of NDEA would be indicated by a figure *1* in black on a *blue* background.

Diet

Before the experiment began, certain contaminant levels were measured in samples of three commercial diets.[27] No aflatoxin, no ochratoxin, and no steam-volatile nitrosamines other than NDMA were detected, but unacceptably high levels of NDMA were found in the Spillers (5–20 ppb) and Oxoid (20–100 ppb) diets. Although lower levels (0–5 ppb) were found in the third diet tested, future supplies of this could not be guaranteed so it was not considered further. The protein source used in the Spillers diet was white fishmeal, samples of which were found to contain 50–250 ppb of NDMA. No steam-volatile nitrosamines or mycotoxins were detectable in soybean meal, an alternative protein source, and Spillers therefore undertook to maintain, for the duration of the study, supplies of their diet with soybean meal substituted for fish meal.

Each fresh batch of diet delivered during the course of the study was analyzed for various contaminants, for each of which a maximum permitted concentration was defined.[28] These were arrived at in the light of an informal estimate of the likely effect of each of the contaminants, together with an estimate of what "None Detected" would really mean in our assays. At no time during the study did any batch of diet attain any of these maximal concentrations, so the mean dietary contaminant concentrations over the whole course of the study must have been quite low.

The diet used was in the form of expanded pellets and was fed *ad libitum* from hoppers on each cage.

Drinking Water

To avoid any interference with the study by the nitrate or nitrite normally present in tap water, distilled water was used throughout. The output from two Fi-stream stills was fed into a 1000-liter high-density polyethylene storage tank. Water was drawn from the tank through food-grade polyvinyl chloride tubing, using a centrifugal pump. The tank was regularly drained and cleaned, using fresh distilled water, to prevent accumulation of any contaminants.

Administration of Nitrosamines

Nitrosamine solutions were prepared weekly, and their concentrations were always checked before use. The volatile nature of the test nitrosamines necessitated the development of procedures that minimized loss, as described below.

To ensure that the animals received full strength solutions, all drinking water bottles were drained each week prior to filling. These quantities of replaced solutions and the excess that was prepared together amounted to a considerable volume of waste nitrosamine solutions. A method of neutralizing this waste was developed (15) and was used throughout the study, and in a parallel study (16).

Preparation of Solutions

All preparation of solutions was carried out in a room within the animal unit that was specifically designated for this purpose. This room housed the stills and the distilled water storage tank described previously, together with a refrigerator for storage of nitrosamines and a hood for the preparation of solutions.

All mixing and dispensing operations were carried out in the hood.

[27] The invaluable assistance of the laboratory of the Government Chemist, and the Tropical Products Institute, in performing these analyses is gratefully acknowledged.

[28] Nitrosamines 5 ppb, nitrate 20,000 ppb, nitrite 500 ppb, aflatoxin plus ochratoxin 5 ppb, polycyclic aromatics 20 ppb, DDT 250 ppb, PCBs 500 ppb, mercury 7 ppb, lead 2 ppb, butylated hydroxytoluene 10,000 ppb.

Small volumes were dispensed with automatic pipets using disposable tips, while larger volumes were dispensed with automatic dispensers. Solution preparation consisted of three dilutions, the first two of which were carried out using glass volumetric flasks. Solutions were mixed with magnetic stirrers. Some of the containers used for the final solutions could not be accommodated in the hood, and for these situations a system was used that permitted handling of the parent solutions to continue in the hood. (This system consisted of a funnel held in a retort stand within the hood, connected by tubing to a screw-on cap which fitted the container for the final solution. A second tube was led back from this cap to the hood to provide an air vent when the bottle was being filled.)

Solutions are prepared as follows.

Stage 1. Stock solutions sufficient to last for 3–4 weeks were prepared at a concentration of 5.3% v/v. These solutions were stored in brown glass bottles at 4°C.

Stage 2. Several liters of a second dilution were prepared at each time of production of solutions, the second dilution containing 1.25 ml of the first dilution per liter and thus having a concentration of 66 ppm, v/v. After this stage the concentration was checked polarographically.

Stage 3. Appropriate volumes of the second dilution (0.5 to 256 ml, depending on the final concentration required) were dispensed into each final solution container and made up to 1 liter with distilled water. These solutions were mixed, using a large magnetic stirrer, for 10 minutes each.

Once all the final solutions had been prepared a small sample of each was taken for analysis. Solutions were deemed acceptable for use if the analyzed concentration was within 10% of that intended, with two exceptions. The dose levels, 1060 and 1590, and 2100 and 2600 were allowed a deviation of only 5% from the intended concentration, to prevent overlap.

All analyses were performed by the Biological Chemistry Department of BIBRA Toxicology International, whose assistance is gratefully acknowledged. The methods used in analysis were polarography for the highest concentrations and gas-liquid chromatography for the lower concentrations.

Dispensing of Nitrosamine Solutions

The containers in which the final solutions were prepared were used to dispense these solutions into the animal drinking bottles. The bottles were filled *in situ* on the racks carrying the cages, by applying 5 psi positive pressure to the head space of the container.

Weekly emptying of the drinking bottles was carried out using a container similar to that used for dispensing but with negative pressure applied to the head space.

Drinking Bottles

The volatile nature of the nitrosamines used in this study necessitated the development of an animal watering system which had minimal leakage. This consisted of a 1.3-liter high-density polyethylene bottle, from the cap at the bottom of which bottle a short length of clear polyvinyl chloride tubing descended and then ended in a horizontal stainless steel needle valve (NKP TV25 Mk II), and into the top of which bottle a syringe needle provided an air vent.

The volume of solution removed by each cage of rats was estimated twice weekly from the difference between two readings on an adhesive scale fixed to the side of the constant-cross-section part of each bottle, the volume removed being determinable to an accuracy of about 10 ml.

Each bottle for each cage was uniquely identified by the same colors and numbers as for the cages (see above), a duplicate being used when one bottle was removed for washing. The bottles were fitted to the racking carrying the cages in an inverted position. The needle valves were held in the racking to locate in eyelets in the rear of each cage, thus avoiding disturbance of the bottles on removal of the cages.

Prior to use the storage characteristics of nitrosamine solutions in these bottles were investigated. On the basis of these results it was decided that fresh nitrosamine solutions would be prepared only once each week.

Observation of Animals and Palpation

Each animal was weighed weekly and at this time was palpated for abdominal masses, particularly those of the liver. Each cage of animals was inspected daily for sick or dead animals.

Palpation

Each rat was palpated once weekly at the time of weighing. Palpation is inevitably a subjective procedure, especially as those performing it were aware of the treatment group each animal was in. However, to avoid too much observer variation a program was devised using a team of three individuals working as shown below:

Palpation schedule: 1, 2, and 3 denote three approximately equal parts into which the study was divided, each containing approximately equal numbers from each treatment

Palpater	Weeks 1, 4, 7, . . .	Weeks 2, 5, 8, . . .	Weeks 3, 6; 9, . . .
A	1	2	3
B	2	3	1
C	3	1	2

If an animal was thought by one of the team to have a palpable lump, a second member of the team was called in to confirm this judgment. When two of the team were agreed that an animal had a palpable lump, that animal was taken immediately for autopsy. Initially, it was found that many of the lumps detected were not liver tumors (see "Results"), so the criteria for accepting lumps as justifying the sacrifice of an animal became more strict during the course of the study. The question of the degree of bias introduced by knowledge of which treatment group palpated animals were in has been considered in both "Results" and "Discussion," and these findings should be considered by future experimentalists who are considering whether or not to use palpation of lumps as an indication for sacrifice.

Sick Rats

Animals that appeared sick were monitored particularly closely. When a sick rat reached a state where it could not reasonably be expected to survive a further 24 h, it was taken for autopsy.

In a number of rats large subcutaneous masses developed. When these reached dimensions sufficient to impede movement seriously, or if ulceration occurred, these animals were taken for autopsy.

Autopsy Procedures

Live animals brought to autopsy were killed by exsanguination from the aorta under deep barbiturate anesthesia. To avoid any possible exchange of tissue samples or other form of confusion, autopsies were carried out sequentially unless numbers (*e.g.*, interim kills) dictated otherwise. The chief objective of the autopsy was defined clearly as the observation and sampling for histology of any macroscopic masses and, if any were observed in animals that had died naturally or that had been sacrificed because of sickness, determination of which, if any, were likely to have been causes of death (or of the sickness that led to sacrifice). Thus, attention was concentrated on the macroscopic examination and description of most tissues, and routine sampling was confined only to the few organs listed in the main text. Histological procedures are also described in the main text.

Safety

Because large volumes of nitrosamine solutions were involved in this study, safety was a major consideration. The unit in which the study was carried out was totally devoted to the nitrosamine study and contained a room for the preparation of solutions and its own cage-wash facilities. The risk attached to handling nitrosamine solutions can conveniently be divided into two areas, skin contact and inhalation. These two problems are considered separately below.

Skin Contact Risk. Protective clothing was provided for every member of staff employed in the unit, consisting of rubber boots, a gown, a hat, a face visor, and thick rubber gloves. This clothing was worn when all operations were being performed in the animal rooms apart from handling the rats, when thin rubber (surgical) gloves were worn. Any member of staff involved in the preparation of nitrosamine solutions was required in addition to wear a full-face gas mask with an appropriate canister attached.

In addition to the protective clothing each member of staff received a set of rules and instructions governing the wearing of the clothing and defining the procedures to be followed in the event of accidental spillage or contact with the nitrosamine solutions. Occasionally some spillage did occur and in these instances the animal room staff was immediately evacuated. The spillage was cleared by an individual in full protective clothing (including a respirator). The method of clearing the spillage was to soak up the solution using paper tissue; this was then sealed in a polythene bag which was sealed in a second bag and taken for immediate incineration. The room was then locked and no access was allowed for at least 1 hr (15 changes of air). On no occasion did any staff member suffer skin contact with any nitrosamine solution.

Inhalation Risk. Perhaps the highest risk area, as for skin contact, was the preparation room, which was the only area in the study where solutions containing more than 17 ppm nitrosamine were handled. This risk was considered adequately met by the use of respirators and hoods in this area.

Throughout the rest of the facility the systems for dispensing the solutions and the drinking bottles attached to the cages were designed to minimize risk in this respect. Analysis of the solutions demonstrated that there was no detectable loss of nitrosamine from either the drinking or the dispensing bottles, in addition the flow rate of air in the animal rooms was rapid (15 changes/h).

A major source of risk in any situation is understaffing, which tends to lead to shortcuts in procedures. Adequate staffing levels were ensured at all times during this study. Similarly, no individual was permitted to work alone in the unit at any time.

Procedures for the neutralization of waste solutions have been described above, and all solid wastes were disposed of by incineration.

Body Weight and Water Intake. These were recorded weekly, but the data were keypunched only for weeks 9, 19, 29, 39, etc. Mean values are given in Appendix Figs. A, B, and C. The increase in mean water intake after 2 years of age is due, at least in part, to the chronic renal disease that affected many of the older experimental animals, as was the decrease in mean body weight. It is clear that no exact correspondence exists between nitrosamine concentrations in ppm and nitrosamine intake in mg/kg/day, but in Section 8 an approximate conversion is suggested that is based on a "typical" intake of 40 ml/kg/day for males and 70 ml/kg/day for females. These approximate figures were chosen to make some *ad hoc* allowance for the small amounts of water that were wasted by the animals and probably provide a reasonable basis for direct quantitative comparison between these data and data from studies in which dose rates were expressed in mg/kg/day.

Acknowledgments

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Note: Public-use copies of the full data are available from R.G.

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